

STUDIES OF COAGULATION AND FIBRINOLYSIS
IN NORMAL AND PREGNANT SUBJECTS

BY

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*"An Inquiry into the properties of the Blood, it is
presumed, will be thought in a particular manner,
interesting, since there is no part of the human
body upon which more physiological reasoning is
founded, nor any from which more inferences
are drawn for the cure of diseases."*

William Hewson 1771

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
STATEMENT IN TERMS OF THE Ph.D. REGULATION OF THE UNIVERSITY OF EDINBURGH	13 iii
SUMMARY	iv
INTRODUCTION	1

SECTION 1.

FUNDAMENTAL CONCEPTS OF COAGULATION AND FIBRINOLYSIS

CHAPTER I.

HISTORICAL DEVELOPMENT OF THE CONCEPTS OF COAGULATION AND FIBRINOLYSIS.	2
A. Coagulation	2
B. Fibrinolysis	6

CHAPTER II.

COMPONENTS OF THE COAGULATION AND FIBRINOLYTIC SYSTEMS.	9
A. Coagulation System:	
Factors I - XIII	9
Cryofibrinogen	18
B. Fibrinolytic System	
(a) Plasminogen	22
(b) Plasmin	23
(c) Plasminogen Activators	24
(d) Inhibitors of Fibrinolysis	29
(e) Fibrin/Fibrinogen Degradation Products	31

CHAPTER III.

THE PHYSIO-PATHOLOGICAL CONCEPTS OF COAGULATION AND FIBRINOLYSIS.

Introduction

1. The Proteolysis of Fibrinogen by Thrombin
2. The Proteolysis of Fibrinogen by Plasmin
3. The Coagulation-Fibrinolytic Equilibrium
4. Disseminated Intravascular Coagulation
5. The Shwartzman Reaction
6. Fibrinolysis and Occlusive Vascular Disease

SECTION 2.

AIMS OF PROJECT AND INTRODUCTORY REVIEW

Aims of Project

Introductory Review

SECTION 3.

LABORATORY METHODOLOGY

CHAPTER I.

MATERIALS.

- A. Equipment
- B. Reagents

CHAPTER II.

COAGULATION TECHNIQUES.

- Global Tests of Coagulation
- Coagulation Factors I - XIII Assay Techniques
- Assay of Contact Activity (Factors XI and XII)
- Heparin Precipitable Fraction Assay

CHAPTER III.

FIBRINOLYTIC ASSAY TECHNIQUES.

1. Plasminogen Assay	94
2. Euglobulin Lysis Time Assay	96
3. Fibrin Plate Assay Technique	97
4. Fibrinogen/Fibrin Degradation Products Assay (I)	98
A. Introduction	98
B. Techniques of Tanned Red Cell Haemagglutination Inhibition Immunoassay	99
C. Standardisation of the T.R.C.H.I.I. Assay	103
5. Fibrinogen/Fibrin Degradation Products Assay (II)	110
A. Rapid T.R.C.H.I.I. Assay Method	110
B. Micro Haemagglutination - Inhibition Assay	111
C. Latex Tests	115

CHAPTER IV.

END POINT RECORDING DEVICES IN COAGULATION WORK.

A. Description of the Coagulometer	121
B. Practical Use of the Coagulometer	124
C. Reproducibility Experiments	125
D. Comments	128

CHAPTER V.

COAGULATION AND THE COMPUTER.

1. Principles of the Computer Programme	131
2. Programme Safeguards	133
3. Comments and Results	135

CHAPTER VI.

STANDARD REFERENCE PLASMAS

1. Pooled Frozen Reference Plasma	141
2. Lyophilised Reference Plasma	144
3. Selected Donors for Standard Reference Plasma	147

SECTION 4.

THE EFFECT OF EXERCISE AND ADRENALIN ON BLOOD COAGULATION AND FIBRINOLYSIS.

CHAPTER I.

THE EFFECT OF EXERCISE ON BLOOD COAGULATION AND FIBRINOLYSIS.	149
Introduction	149
Subjects and Experimental Design	154
Results	158
Discussion	164
Summary	176

CHAPTER II.

THE EFFECT OF ADRENALIN AND BETA-ADRENERGIC BLOCKAGE ON THE COAGULATION AND FIBRINOLYTIC SYSTEMS.	177
Introduction	177
Project Aims	181
Subjects and Experimental Design	182
Results	186
Discussion	196
Summary	203

SECTION 5

COAGULATION, FIBRINOLYSIS AND PREGNANCY.

INTRODUCTION	204
--------------	-----

CHAPTER I.

NORMAL LEVELS OF HAEMOSTATIC FACTORS IN PREGNANCY.	205
A. Introductory review of coagulation and fibrinolytic changes occurring in normal pregnancy	205

B. Experimental study of the normal levels of selected coagulation and fibrinolytic parameters in pregnancy	207
1. Subjects and Methods	207
2. Results	209
3. Discussion	216
4. Summary	227

CHAPTER II.

THE EFFECT OF MODERATE EXERCISE ON THE FIBRINOLYTIC SYSTEM THROUGHOUT PREGNANCY.	228
Introduction	228
Subjects and Methods	230
Results	231
Discussion	236
A. Plasminogen Activator Baseline and Response Study	236
B. Fibrin Degradation Products Study	241
C. Heparin Precipitable Fraction Study	242
Summary	243

CHAPTER III.

SURGERY, COAGULATION AND FIBRINOLYSIS IN BOTH PREGNANT AND NON-PREGNANT SUBJECTS.	244
Introduction	244
Methods	246
A. Selection and Management of Subjects	246
B. Blood Sampling Methods	248
C. Assay Techniques	249
Results	250
A. Gynaecological Operations	250
B. Caesarian Section Operations	255
C. Comparisons of Operative Results of Normal and Pregnant Subjects	263

Discussion	266
A. Intra-operation Changes	266
B. Post Operation Changes	275
Summary	284

CHAPTER IV.

HAEMOSTATIC PROBLEMS OF PREGNANCY.	286
Introduction	286
Methods	287
1. Abruptio Placentae	288
2. Intra-Uterine Death	299
3. Amniotic Fluid Embolism	309
4. Termination of Pregnancy	326
5. Secondary Post-Partum Haemorrhage	331
Discussion	337
Summary	339

SECTION 6.

FINAL COMMENTS.

FINAL COMMENTS.	340
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APPENDIX

A. Collection and Storage of Blood Specimens	347
B. Statistics	347
C. Clinical Details of Surgical Patients	355
D. General Reagents	361
E. Cleaning and Siliconization of Glassware	363
F. Electrocardiograph Reports - Middle-Aged Volunteers	365

REFERENCES

REFERENCES	366
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Some studies reported in this thesis have been the subject of published articles.

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SUMMARY

In this thesis, the development, refinement and standardisation of a wide range of coagulation and fibrinolytic assay techniques are described. Particular attention was directed towards the assay of Factor VIII, cryofibrinogen and fibrin/fibrinogen degradation products (F.D.P.). A new type of coagulation end point recorder was investigated and the use of the Atlas computer in the analysis of results is outlined. In addition, a study was made of various standard plasma preparations for the control of coagulation factor assays.

Using these laboratory techniques, the effect of severe and moderate exercise, adrenalin infusions and beta-adrenergic receptor blockage on selected parameters of the coagulation and fibrinolytic systems, in normal volunteers, were studied. No correlations were observed between parameter changes in either system, and contrary to some reports, Factor XII was not increased post exercise. Some subjects exhibited a low fibrinolytic response to the exercise procedures, confirming previous work from this laboratory, but the coagulation response in these individuals did not appear to differ in any constant manner from the normal pattern. The adrenalin induced changes in Factor VIII were completely blocked by Propranolol, but this drug only partially lowered the response to adrenalin of plasminogen activator. The new beta-adrenergic blocker drug, ICI 50172, was found to have only minimal effects on the two systems. These results are fully discussed in the light of presently available concepts of the haemostatic mechanism.

In the studies on pregnant subjects, parameters of coagulation and fibrinolysis throughout the gestational period were measured. One new finding was that of a significant increase of serum F.D.P. in late pregnancy. It was also found, in another study, that the reactivity of the fibrinolytic system to an exercise stress in the third trimester was greatly impaired in some subjects. Further coagulation and fibrinolytic studies in pregnant and non-pregnant patients undergoing intra-abdominal surgical operations, demonstrated that significant changes occurred in euglobulin lysis activity, fibrinogen and F.D.P. levels. The application of specific coagulation and fibrinolytic tests to clinical haemostatic emergencies of pregnancy is described in the final section. All these studies have provided a basis for a fuller discussion in each section, of concepts such as the Shwartzman reaction, disseminated intravascular coagulation, and haemorrhage and thromboembolism.

It is concluded that although some of the physio-pathological interrelationships of coagulation and fibrinolysis are now becoming clarified, many problems still remain, and these may only be resolved when improved methodology becomes available. The extension of work reported in this thesis may aid in the elucidation of important aspects of haemorrhage and thrombosis.

INTRODUCTION

A fundamental alteration has now taken place in the approach of research workers to blood coagulation and fibrinolysis. In the past much exacting work had been directed to the simple identification of pathological disturbances of haemostasis, and from this approach there has arisen a clinically orientated concept of the two systems as well as many therapeutic advances. Recent investigations, however, have been increasingly directed towards the pathophysiology of these systems with three main goals in view, namely, the purification and characterisation of specific components, the establishment of the exact mechanism of interactions by which these factors integrate to form and dissolve fibrin clots, and the study of the theory of a dynamic balance between these two functionally antagonistic systems.

The problems of investigation have been formidable, but as new laboratory techniques have evolved, a much more complete understanding of the complexity of the systems governing blood fluidity has arisen. This physiological research has been stimulated by the realisation that one key to the problems of atherosclerosis and thrombosis may lie in the elucidation of these mechanisms of haemostasis. Yet despite progress, our accumulated knowledge remains limited and many questions remain unanswered. The work in this thesis therefore is presented as a study of problems relating to physiological and pathological coagulation and fibrinolysis utilizing both well established and recently developed laboratory techniques.

CHAPTER I.

FUNDAMENTAL DEVELOPMENT OF THE CONCEPTS OF COAGULATION AND FIBRINOLYSIS.

A. Coagulation.

Dr. William Harvey (1771) was perhaps the first research worker to clarify some of the basic facts of blood coagulation. In a series of experiments, a few of which are the prototype of techniques used to-day, he demonstrated that the coagulative portion of blood resided in the plasma and not in the red cells.

SECTION 1

FUNDAMENTAL CONCEPTS OF COAGULATION AND FIBRINOLYSIS.

Most of the research was important in understanding clotting. Further progress was not made until 1845 when Andrew Buchanan reported that animal fibrin would promote the coagulation of hyaline fluids and in 1855 he was the first to report that the active coagulant was a protein belonging to the globulin. Schmidt (1872) confirmed these observations and called the active coagulant thrombin which, he hypothesized, was derived from an inactive precursor, prothrombin. Virchow (1858), led by this idea postulated the existence of fibrinogen and this was subsequently isolated by Denis in 1869.

The stage was thus set for the classical observations of Minot (1905) upon which the modern theory of coagulation is still

CHAPTER I.

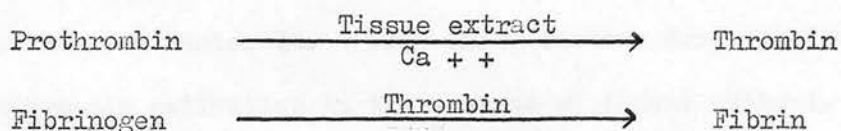
HISTORICAL DEVELOPMENT OF THE CONCEPTS OF COAGULATION AND FIBRINOLYSIS.

A. Coagulation.

Dr. William Hewson (1771) was perhaps the first research worker to clarify some of the basic facts of blood coagulation. In a series of experiments, a few of which are the prototype of techniques used to-day, he demonstrated that the coagulative portion of blood resided in the plasma and not in the red cells, that clotting of blood was not due to changes in temperature or the absence of motion and that stagnation of blood within vessels was important in intra-vascular clotting. Further progress was not made until 1845 when Andrew Buchanan reported that washed fibrin would promote the coagulation of hydrocoele fluids and in 1879 Gamgee was able to report that the active coagulant was a protein belonging to the globulins. Schmidt (1872) confirmed these observations and called the active coagulant thrombin which, he hypothesised, was derived from an inactive precursor, prothrombin. Virchow (1856), had by this time postulated the existence of fibrinogen and this was subsequently isolated by Denis in 1859.

The stage was thus set for the classical observations of Morawitz (1905) upon which the modern theory of coagulation is still

based. These may be written :-



As with many theories, it was not generally accepted, and it was not till 30 years later, following the experiments by Quick (1935) and Warner, Brinkhous and Smith (1936), that its validity was established and tests derived for measuring its components. However, experience soon demonstrated that anomalies incompatible with the theory occurred and the presence of other coagulation factors was suspected.

The first to be discovered was Factor V by Owren (1947) and this was followed by Factor VII by Owen and Bollman (1948); Mann and Hurn (1950) and Alexander et al. (1951). Factor X was identified much later (Telfer et al., 1956; Hougie et al., 1957). These factors were necessary in clotting mixtures activated by tissue thromboplastins.

It had been realized much earlier that the Morawitz theory did not explain the clinical state of haemophilia and it was therefore concluded that there was an alternative pathway for the activation of prothrombin. It became evident that haemophilia was due to a lack of a specific clotting factor and the defect was localised to the globulin fraction of plasma by Patek and Taylor (1937) and subsequently labelled "antihaemophilic factor" or Factor VIII. Pavolovsky (1947) later made the observation that blood from two haemophilic patients were mutually corrective and based on this finding, separate investigations by Aggeler et al. (1952) and Biggs et al. (1952) clearly demonstrated the existence of a hitherto undescribed coagulation factor,

named at that time 'plasma thromboplastin component' (P.T.C.) or "Christmas Factor", and now known as Factor IX. Both these factors were apparently important in prothrombin activation in the absence of tissue extract. In 1953 another new clotting factor, plasma thromboplastin antecedent (Factor XI), absent in certain patients, was described (Rosenthal, 1953), and this was followed by the discovery of Factor XII, in 1954, by Ratnoff. These latter two factors, both active in the early stages of coagulation, and named the 'contact factors' were shown to be essential for the development of prothrombin activator. A further factor, Factor XIII, essential for the polymerisation of fibrin to form insoluble fibrin was detected in 1958 (Lorand et al.)

However, the identification of these separate components did not explain their mechanism of reaction to form prothrombin activator. Initially, the clotting sequence was arbitrarily subdivided into an "extrinsic" and an "intrinsic" pathway, the former initiated by tissue extracts, and the latter by surface contact. From considerable deductive experimental work, it became apparent that process of clotting appeared to consist of a series of stages in a chain reaction in which coagulation factors were acting as proenzymes for other factors. Such a scheme was outlined by Macfarlane in 1964, and is now referred to as the "cascade theory" of blood coagulation (Fig. 1:1).

This concept unifies much of the available information on coagulation and has been accepted by most workers. A more biochemical explanation of coagulation has been evolved by Seegers et al. (1962) based upon the autocatalytic conversion of prothrombin to its various enzyme derivatives. This theory, although of considerable theoretical interest

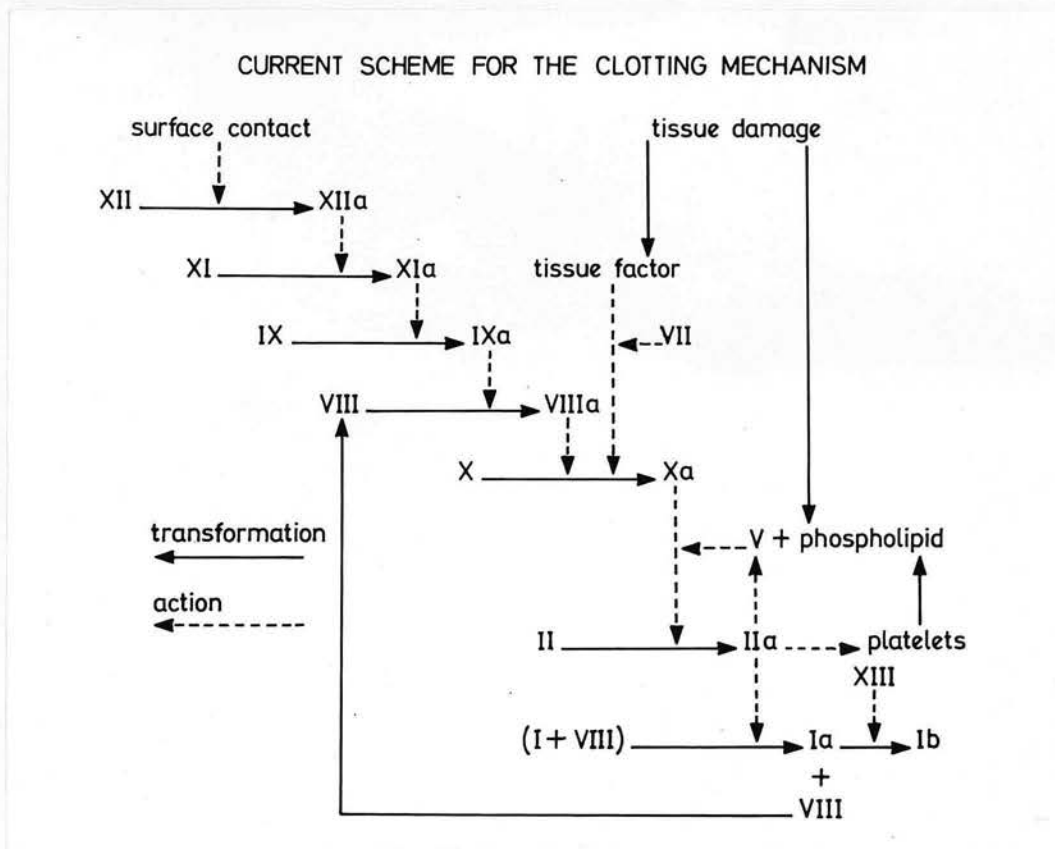


Fig. 1:1. Current Scheme for the Clotting Mechanism

does not lend itself so readily to the study of abnormal haemostasis and has not generally been accepted by clinicians. However, present concepts of coagulation do not yet fully explain all the phenomena observed and further advances in knowledge can be expected.

B. Fibrinolysis.

The earliest known observation relating to fibrinolysis was made by Morgagni in 1761 who remarked on the post mortem fluidity of blood in cases of sudden death. John Hunter in 1786 also observed this phenomena but it was not till 1838 that Denis described the actual lysis of whole blood. Further progress was slow but in 1893 Dastre proposed the word fibrinolysis. Nolf (1908), with considerable foresight, postulated that fibrin deposition and removal were in a state of balanced equilibrium within the circulation, but little further interest was taken until the discovery of streptococcal lysis of fibrin (Tillet and Garner, 1933). Subsequent work by Milstone (1941), and Christensen and Macleod (1945) characterised the components of the fibrinolytic system, providing a basis for further research into lytic mechanisms.

Interest in the fibrinolytic system is a comparatively recent development, as compared with the coagulation system. It was soon realised that fibrinolysis was a basic physiological process, which was operative not only in the vascular system but also in tissues, and other body fluids. It was postulated that fibrinolysis was active in preserving the patency of various types of excretory ducts (Astrup and Sterndorff, 1955), in the physiology of endometrial activity (Albrechtsen, 1956), and

SIMPLIFIED SCHEME OF THE FIBRINOLYTIC SYSTEM

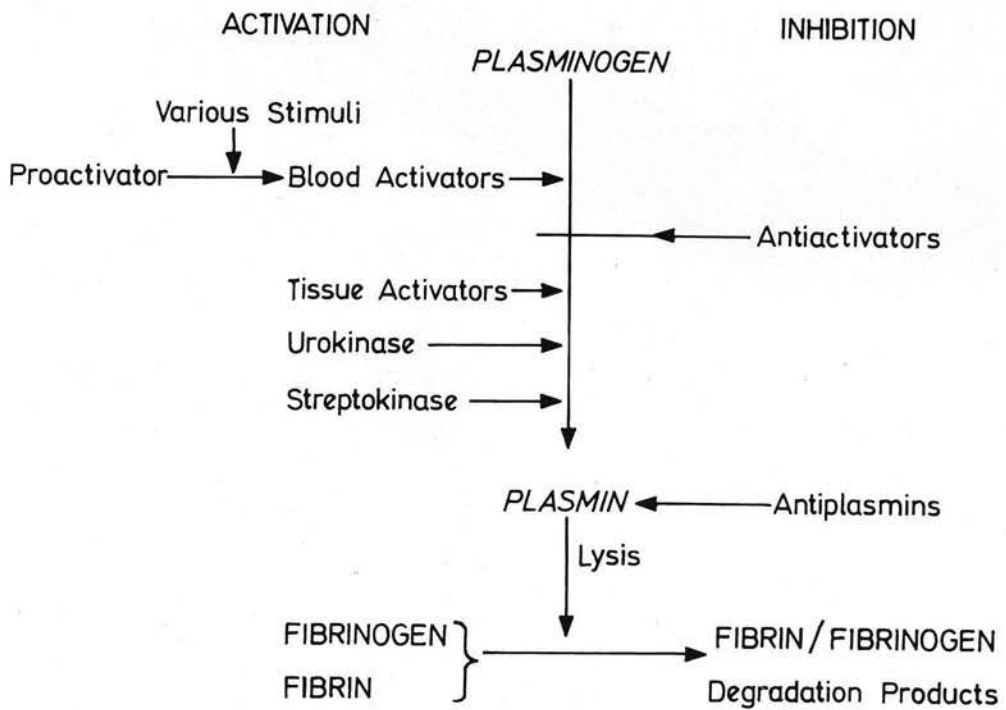


Fig. 1:2. Simplified Scheme of the Fibrinolytic System.

in wound healing (Astrup, 1955). A major function ascribed to the fibrinolytic system was the clearance of fibrin from the vascular bed (Astrup, 1956).

A modern concept of the fibrinolytic system is summarized in Fig. 1:2. This physiological system is constantly active in normal circumstances and is held in check by potent inhibitor systems. The formation of plasmin is normally quickly neutralized by antiplasmins, but if excess plasmin is produced, it may transiently appear in the blood stream with marked proteolytic effects on other blood proteins.

In Section 1:3 a fuller consideration will be made of the proposed inter-relationships existing between coagulation and fibrinolysis.

CHAPTER II

COMPONENTS OF THE COAGULATION AND FIBRINOLYTIC SYSTEM

A. Coagulation.

(a) Factor I (Fibrinogen).

Fibrinogen, a plasma protein of molecular weight 340,000-360,000 (Caspary and Kekwick, 1957; Blombäck and Laurent, 1958), is probably produced in the liver (Tocantins, 1938), by the liver parenchymal cells where it has been identified by fluorescent antibody studies (Barnhart and Anderson, 1962). Electron microscopy studies indicate that the molecule consists of three globules in a row connected by thin filaments (Hall and Slayter, 1959) and the dimensions of these components had been established (Blombäck, 1967). The analysis of human fibrinogen shows the presence of all the common aminoacids and the difference between it and bovine fibrinogen is small (Henschen and Blombäck, 1964). The molecule also resembles the structural proteins of skin (epidermin) and muscle (myosin) (Bailey et al., 1943). It has the built-in property of "self assembly" after thrombin activation, to form orderly molecular aggregates recognizable as fibrin.

Fibrinogen is a sensitive substrate for the proteolytic enzymes of blood, notably thrombin and plasmin, and both these reactions are discussed in a later section (Section 1:3). It is not known if a continuous low rate of clotting and lysis contributes to the metabolism of fibrinogen turnover, but in pathological states of plasmin release,

marked changes in fibrinogen levels can occur from both disseminated coagulation and fibrinolysis (Schneider, 1964). Normally, the concentration of fibrinogen in the blood is maintained between narrow limits. Its half life is between 2.1 and 3.8 days (Macfarlane et al., 1964), and 75% of total body fibrinogen is contained in the intravascular compartment (Adelson, 1965). Fibrinogen turnover estimated in grammes per day is approximately 1.5 - 5.0g but this may be apparently increased in certain situations of relative hypercoagulability (Galewski et al., 1965).

Numerous methods are available for measuring the concentration of fibrinogen. These include estimating the amount of fibrin evolved after the addition of thrombin (Ratnoff and Menzies, 1951); salt precipitation methods (Goodwin, 1961); heat precipitation methods (Stirland, 1956); electrophoretic determinations (Berkes et al., 1956), and an immunochemical method using sensitised tanned red cells (Fox, 1964). Assays measuring the thrombin clottable protein have proved the most reliable in clinical situations.

(b) Factor II (Prothrombin).

Prothrombin is a plasma protein, produced in the liver parenchymal cells, migrating electrophoretically in the α_2 fraction and having a molecular weight of 70,000 (Aronson and Preiss, 1962). It has been studied extensively by Seegers (1967) whose biochemical approach to coagulation has attracted considerable interest. It is a relatively stable protein, is closely associated with the activity of Factors VII, IX and X, and is present in Cohn alcohol Fraction III, from which it can be concentrated.

Activation of prothrombin leads to the generation of thrombin and much of the current research work in coagulation is orientated towards elucidation of the factors which convert prothrombin to thrombin. Prothrombin deficiency as a congenital defect is very rare but acquired partial deficiencies (anti-coagulant therapy and liver disease) are relatively common.

Thrombin, the active enzyme from its stable precursor prothrombin, can be purified from Cohn alcohol Fraction III (Miller et al., 1965), and when concentrated has high stability on freeze drying. Chromatography on Sephadex G-100 has been used to estimate the molecular weight of thrombin to lie between 26,000 and 35,000 (Miller et al., 1965; Lanchantin et al., 1963). Chemical analysis yields much lower values with minimum values of 8,000 - 9,000 g (Iaki and Gladner, 1964). The amino acid composition of thrombin has been determined, twenty acids having been identified (Miller et al., 1959).

It is a highly specific proteolytic enzyme but will hydrolyse not only a fibrinogen substrate but also p-toluene sulphonyl-L-arginine methylester (TAME) (Troll et al., 1954), lysine ethylester and methylester and the lysylalanine bond in the oxidized B chain of insulin (Scheraga, 1958).

It is adsorbed readily onto glass (Seegers et al., 1952) from which it can be removed with difficulty. Its activity is very sensitive to salt concentration, colloidal substances and calcium concentration (Seegers and Smith, 1942), and it tends to be unstable in low concentrations.

In vivo, its activity is held in check by various powerful anti-thrombins only one of which is generally accepted as being of physiological

importance (Anti-thrombin III).

(c) Factor III (Tissue Extract).

Considerable investigation still has not clarified the nature of tissue extracts or thromboplastin. The consensus of opinion is that the active agent in thromboplastin preparations, whether derived from brain, lung or placenta, is a lipoprotein, in which the lipid part is closely related to cephalin (Chargaff et al., 1944) and has a high molecular weight. It converts prothrombin to thrombin in the presence of Factors V, VII and X, calcium chloride and phospholipid.

(d) Factor IV (Calcium).

Calcium is generally required in the coagulation process but its exact mode of action has not been clarified.

(e) Factor V (Proaccelerin).

Factor V is a trace plasma protein that is essential for the rapid conversion of prothrombin to thrombin in the presence of a brain extract and calcium. It is very labile and attempts at purification have been difficult (Lewis and Ware, 1953; Surgenor et al., 1961; Aoki, 1963). A preparation made by Esnouf (1963) was homogeneous on ultracentrifugation and its molecular weight was estimated to be 290,000 which was in agreement with Lewis (1964) who, using a Sephadex gel technique found a molecular weight for Factor V of over 200,000. The exact site of its bio-synthesis is not known with certainty but work by Barnhart and Ferar (1963) using fluorescent marker techniques suggest that the liver may be at least one site.

It is unstable in stored plasma and is very susceptible to the action of plasmin (Johnson et al., 1957; Ouchi et al., 1961). Deficiency of this factor results in a moderate bleeding tendency, and relatively few cases have been described (Biggs and McFarlane, 1962).

(f) Factor VII (Proconvertin).

Factor VII, a β globulin, which is closely related to coagulation Factors II, IX and X is produced in the liver. It is an accelerator in the formation of prothrombin activator by tissue extract (de Vries et al., 1949), and is present normally in serum. Little is known of its biochemistry due to its difficulty of separation from the other clotting factors (II, IX and X) and lack of homogeneity on purification, but its molecular weight has been estimated as lying between 50,000 and 100,000 (Lewis, 1964).

Deficiency causes a rare haemorrhagic state, which has also been recognised in a colony of Beagle dogs (Garner, 1967).

(g) Factor VIII (Antihæmophilic Factor).

Antihæmophilic factor is probably a trace protein of normal blood the lack of which results in a clinical bleeding diathesis called hæmophilia. Patek and Taylor (1937) were the first to show that addition of a small amount of globulin to hæmophilic blood corrected the defect and much subsequent work has been directed towards identification and purification of the active factor. Factor VIII migrates on electrophoresis with either the α_2 (Barkhan et al., 1963) or β globulins (Van Creveld et al., 1956; Shinowara, 1964). The amino acid composition is not known but some authorities have suggested that Factor VIII may be a lipoprotein (Blomback

et al., 1962a; Simonetti et al., 1964). Its molecular weight has been assessed as over 200,000 (Michael and Tunnah, 1966).

Factor VIII is usually found in close association with fibrinogen from which it can be separated by curtain electrophoresis and other techniques. (Pavlovsky et al., 1961; Blomback et al., 1962; Rizza et al., 1965). It is unstable on storage and very sensitive to the action of plasmin (Wagner et al., 1959). In vitro experiments have demonstrated that there may be a link between the lysis products of fibrinogen and Factor VIII activity (Triantaphyllopoulos and Triantaphyllopoulos, 1967).

In vivo, the half life of Factor VIII is between 8 and 15 hours (Biggs and Denson, 1963; Pool et al., 1967), and the level above which normal haemostasis can be maintained is near 30% of normal plasma activity. Its site of production is not known; but recent work implicates the spleen and particularly the reticulo endothelial cells of the body (Webster et al., 1967).

(h) Factor IX (Christmas Factor).

Factor IX migrates on electrophoresis with the β globulins, and is probably also synthesized in the liver. It is closely related in structure to Factors II, VII and X, and has a molecular weight of between 100,000 and 200,000 (Lewis, 1964). It is required for the formation of blood prothrombin activator and its activity in vitro increases during clotting (Ratnoff and Davie, 1962; Cattan and Denson, 1964). Its deficiency results in a severe bleeding diathesis, closely similar to Factor VIII deficiency.

(i) Factor X (Stuart Prower Factor).

The activity of Factor X can be found on electrophoresis in the α globulin fraction, has a molecular weight between 50,000 and 100,000 (Lewis, 1964), and is present in plasma and serum in trace amounts. Its chemical structure is similar to others of the prothrombin complex, and it is also produced in the liver. It can be separated only with difficulty from Factors II, VII and IX, but pure preparations which are antigenic in rabbits have been obtained (Denson, 1967). The factor is named after the first two patients discovered in whom a deficiency was detected.

(j) Factor XI (Plasma thromboplastin antecedent).

This factor has not been satisfactorily purified but migrates on electrophoresis between the β and γ globulins, and one estimation of the molecular weight (Lewis, 1964) is between 100,000 and 200,000. It is present in plasma and serum and its absence causes a mild haemorrhagic disorder. It is converted to the activated form by activated Hageman factor, which in turn can activate Factor IX (Ratnoff and Davie, 1962).

(k) Factor XII (Hageman Factor).

Human Hageman factor, a protein present in plasma in trace amounts, migrates upon electrophoresis between the β and γ globulins (Haanen et al., 1961), and has a molecular weight between 100,000 and 200,000 (Lewis, 1964). A little is known of its chemical nature as Speer et al. (1965) has shown that end group analysis of the molecule reveals the presence of arginine and methionine. Complete amino acid analysis also demonstrated a high content of serine and glutamic acids,

and it is possible that Hageman Factor can be classed as glycoprotein as it also contains hexoses, hexosamines and sialic acid (Schoenmakers et al., 1965).

Knowledge of the existence of Hageman Factor arose from the discovery of a patient, Mr. Jack Hageman, who was found to have a greatly prolonged clotting time but without any evidence of a haemorrhagic diathesis. Ratnoff (1954) was able to show clearly that the abnormality was due to the deficiency of a single clotting factor which was therefore named Hageman Factor, and its deficiency, Hageman trait. Many other patients exhibiting the coagulation anomaly have now been described and statistical analysis has shown that Hageman trait is inherited as an autosomal recessive characteristic (Margolis, 1956; McCain, 1959; Veltkamp, 1967).

The presenting haematological picture of Hageman trait is characteristic. All tests involving the intrinsic pathway are abnormal and small amounts of normal plasma or serum will correct the deficit (Jim and Goldfein, 1957). The deficit is one confined to the initial stage of activation of the intrinsic pathway of coagulation. Contact activation of Hageman factor in vitro can be achieved by glass, kaolin, diatomaceous earth, charcoal, ellagic acid and many other substances (Ratnoff, 1966). In vivo, the stimulus for activation of Hageman Factor is not known although Niewiarowski's observations (1964) that preparations of bovine collagen fibrils activate Hageman Factor suggests one method of action. The Hageman Factor is apparently adsorbed onto the activating surface, converting Factor XI into an activated form perhaps by an enzymic process (Ratnoff et al., 1961).

Considerable interest, however, has been aroused in other postulated biological functions of Hageman Factor. These include an effect on capillary permeability (Spector, 1957), the kinin forming system (Armstrong et al., 1954) and on fibrinolysis (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961, 1962b; Aznar et al., 1964). However, the significance of activation of Hageman Factor within the body is still unknown, as subjects with this trait do not appear to be particularly handicapped by the inability of their blood to react to contact activation, kinin formation or fibrinolysis.

(1) Factor XIII (Fibrin Stabilizing Factor).

Fibrin stabilizing factor (F.S.F., Factor XIII) is a plasma protein, migrating electrophoretically with the α_2 globulins and having a molecular weight of about 350,000 (Loewy et al., 1961). Patients with a deficiency of this protein exhibit a severe bleeding diathesis and their fibrin clots lack the typical cross striation normally seen on electron microscopy (Duckert et al., 1960). F.S.F. may also be connected with the normal growth of fibroblasts (Beck et al., 1961). It can be found in a wide variety of tissues including brain, kidney, lung, liver and muscle (Tyler and Lack, 1964), and is also present in red blood cells and on the surface of platelets (Buluk and Malofiejew, 1955; Luscher, 1957; Kiesselback and Wagner, 1966).

Fibrin clots, stabilized by F.S.F. are insoluble in 5 M urea solution (Laki and Lorand, 1948) and in monochloroacetic acid and methods of assay have been devised based on these properties (Duckert et al., 1960; Sig, 1966; Das, 1968). Tyler and Lack (1965) have shown that

fibrinolysis may be affected by F.S.F. as clots formed in the absence of F.S.F. are more susceptible to lysis, perhaps by altering the ability of fibrin to adsorb plasminogen activator.

(m) Platelets.

An active substance known as Platelet Factor 3 has been extracted from platelets by Alkjaersig et al. (1955) and others. Such phospholipid preparations are known as partial thromboplastins and can also be extracted with chloroform from organs such as the brain (Bell and Alton, 1954). Phospholipid is essential for normal coagulation via the 'intrinsic' system.

(n) Cryofibrinogen (Heparin Precipitable Fibrinogen, H.P.F.)

Cryofibrinogen is a clottable protein with physical and chemical properties similar to fibrinogen, but exhibiting the property of reversible precipitation on cooling (Lipinski, 1965). Its precipitation is enhanced by the presence of small quantities of heparin; it is then known as the Heparin Precipitable Fraction (H.P.F.) of plasma. Small quantities of cryofibrinogen can also be demonstrated in citrated or exalated plasma (McKay, 1964).

The first reports of a cold precipitable form of fibrinogen in the plasma of patients was made in 1946 by I. Morrison. He detected a substance named "Contractinogen" that precipitated when some plasmas, particularly from those patients who had high erythrocyte sedimentation rates, were cooled. In 1948 a similar protein was found in normal plasma (P. Morrison et al.). The first extensive study of cryofibrinogen as

measured by the H.P.F. technique was made by Thomas (1954). Heparinised chilled plasma from rabbits submitted to an injection of endotoxin was found to contain a redissolvable precipitate and electrophoretic studies demonstrated its similarity to fibrinogen. Smith (1956, 1957), working in conjunction with Thomas, investigated H.P.F. in both normal subjects and patients with a variety of diseases. He was able to demonstrate H.P.F. in the plasma of normal individuals and in patients with inflammatory and necrotizing disorders. A gradual increase of H.P.F. throughout pregnancy, as well as low levels in newborn infants, was also noted. Reports soon followed of cryofibrinogenaemia in varied pathological states. It was demonstrated in diabetes, duodenal ulcer, and myocardial damage (Ritzmann et al., 1963); leukaemia and meningitis (Jager, 1962); lung cancer (Korst et al., 1955); cancer of the stomach, ovary and prostate (Kalbfleisch et al., 1960; Kessel, 1960); and in multiple myeloma (Henstill and Feinstein, 1957). A form of primary idiopathic cryofibrinogenaemia was also reported with clinical symptoms of purpura, vascular occlusions and finger tip necrosis (Harville, 1966; Ruiter, 1962; Harmel-Tourneur and Kalis, 1964; Robinson et al., 1966). McKay and Carey (1966), suggested that cryofibrinogen (H.P.F.) could be found in the blood of patients with diseases characterised by the deposition of fibrin either extra- or intravascularly, and regarded cryofibrinogenaemia as a hall-mark of disorders accompanied by intravascular coagulation.

Despite considerable investigation, the exact nature of this cryofibrinogen remains uncertain. One source of confusion is the variety of cold precipitable proteins described as cryofibrinogen. Heparin will

precipitate a form of cryofibrinogen from plasma, but Smith and von Korff (1957) found that other heparin-like polysaccharides, e.g. sulphates of alginic and polymannuric acids and chondroitin sulphate, produced similar precipitates. Another substance very similar to H.P.F. is the Fibrinogen B. described by Lyons (1945). This is a coagulable protein precipitated from plasma under the influence of certain derivatives of naphtho-quinone. This protein was not demonstrated in normal subjects but occurred in large amounts in similar types of disorders which are usually accompanied by increased cryofibrinogen (Dunn et al., 1949). Cryofibrinogen can also be demonstrated using other anticoagulants, e.g. E.D.T.A., citrate and oxalate, and using plasma or pure fibrinogen similar complexes can be formed by the use of alcohol (Godal and Abildgaard, 1966) and chondromucoprotein (Anderson, 1963). It is probable that all these substances represent combinations of fibrinogen, fibrinogen derivatives and other proteins, differing only in the proportions of the various components.

Electrophoretic studies of cryofibrinogen (H.P.F.) have shown that its electrophoretic mobility is identical to that of fibrinogen (Smith and von Korff, 1957) and that traces of other proteins are usually present, such as prothrombin and proaccelerin (Kalbfleisch and Bird, 1960). On ultra centrifugation of purified cryofibrinogen two peaks of constant sedimentation were observed and Korst and Kratochvil (1955) suggested that the protein was a form of partially polymerised fibrinogen, either in combination with fibrinogen or other plasma proteins, and was produced by the action of thrombin in vivo.

Shainoff and Page (1960, 1962) prepared a form of cryofibrinogen

named cryoprofibrin from rabbit plasma by use of a Cohn alcohol fractionation process. According to their experience, subthreshold amounts of thrombin, constantly being formed intravascularly, split fibrinopeptide A from fibrinogen. This altered fibrinogen then polymerises with unaltered fibrinogen to form a complex soluble at high concentrations of fibrinogen at 37°C, but insoluble at 4°C, this being the definitive cryoprofibrin. Their studies have been most extensive and they further showed that if the complex polymer formed exceeded more than 10-15% of the total fibrinogen concentration, as in some abnormal conditions, the solubilising capacity of fibrinogen was exceeded and fibrin conjugates of high molecular weight were precipitated (Shainoff and Page, 1964). This work was confirmed by Lipinski et al. (1964), and extended to show that cryofibrinogen was not simply a fibrinogen-fibrin complex requiring the presence of calcium for its precipitation but that fibrinogen degradation products were also present. These degraded fragments of the fibrinogen molecule, produced by the action of plasmin, were capable of co-polymerising with fibrin monomers.

Particular interest has been re-aroused in cryofibrinogen, as related to the concept of disseminated intravascular clotting. Thomas's first work on cryofibrinogen was in endotoxin treated rabbits and the appearance of H.P.F. paralleled the incidence of the generalized Shwartzman reaction. As a Shwartzman-like reaction is thought to occur in man (McKay, 1962), it has been hypothesised (McKay, 1965), that "the appearance of large amounts of (heparin) cold precipitable fibrinogen (cryofibrinogen) is a common feature of disseminated intravascular clotting". However, few reports have become available in which cryofibrinogen has been assayed in humans

demonstrating features of the generalised Shwartzman reaction, and thus this concept, in man, has yet to be fully established. In vitro work, however, clearly shows that if suboptimal amounts of thrombin are added to plasma, i.e. insufficient to cause coagulation, cryofibrinogen formation is enhanced (Heinrich et al., 1963; van der Weerdt and Vreeken, 1965), but the in vivo significance of these observations are not yet clear.

Thus the exact significance of cryofibrinogen awaits fuller investigation. In this thesis, it has been assumed that the measurement of H.P.F. reflects to some extent, changes in cryofibrinogen. However, the qualification must be made that measurement of H.P.F. may not necessarily reflect alterations in the cryoprotibrin of Shainoff (1962) which, from his results presented, is more likely to be the relevant measurement to assess in vivo thrombin activity (Shainoff, 1967). Cryoprotibrin assays are unfortunately much more difficult and require ultracentrifugation techniques.

B. Fibrinolytic System.

(a) Plasminogen.

Plasminogen, the enzyme precursor of plasmin, is a β globulin with a molecular weight of either 83,000 (Davies, 1960) or 143,000 (Robbins and Summaria, 1963), and is present in the euglobulin fraction of plasma (Milstone, 1941). It can be partly purified by treatment of Cohn Fraction III (Kline, 1953) and further purification has been achieved by chromatography (Hagan, 1960; Wallén and Bergström, 1960; Robbins and Summaria, 1964). There is some evidence that human plasminogen may be heterogeneous in nature, perhaps depending on its method of purification

(Rybak and Petakova, 1963; Alkjaersig, 1964), and this could explain the variability in the molecular weights found.

Plasminogen is probably produced in the liver (Sherry, 1965) although Barnhart and Riddle (1963), from immunofluorescent studies, suggest that bone marrow cells and eosinophils may be the main source of supply. The control mechanism of its production is unknown, although steroid metabolism may be relevant (Pilgeram et al., 1964).

Plasminogen has a strong affinity for fibrinogen and most purified fibrinogen preparations are contaminated to a greater or lesser degree. However, the fibrinogen can be separated by gel filtration (Berg and Korsan-Bengtsen, 1963). Plasminogen may be adsorbed to fibrin in vivo thus promoting optimal conditions for fibrin lysis (Sherry, et al., 1959b).

Plasminogen is widely distributed in tissues, and is present in circulating blood at a level of 0.1 - 0.2 mgm. per ml. of plasma.

(b) Plasmin.

Plasmin, the fibrinolytic protein derived from its inactive precursor plasminogen, is a potent enzyme which will digest fibrin, fibrinogen, several of the coagulation factors (II, V and VIII), some components of complement, ACTH, growth hormone and glucagon, as well as proteins from other sources such as casein (Sherry et al., 1959a). Its action is normally curbed by the presence of various antiplasmins (Christensen and Macleod, 1945; Norman, 1966).

It has a molecular weight of about 107,000 (Shulman et al., 1958), but its structure awaits clarification. Plasmin probably splits the arginine and lysine bonds in proteins (Troll and Sherry, 1955). It digests

fibrin and fibrinogen at equal rates (Ratnoff, 1953), and its action in the proteolysis of fibrinogen will be considered further in a later section.

(c) Plasminogen Activators.

These are substances which will convert plasminogen to plasmin.

(i) Physiological Activators.

Plasminogen activators are widely distributed in body organs and fluids, and are named as to their site of origin.

Tissue Activators.

Most human tissues contain variable amounts of plasminogen activators, apparently located in the cytoplasm of cells (Astrup, 1951), and with the highest activity being found in the lysosomal fraction (Lack and Ali, 1964). The placenta and liver are notable for the lack of these enzymes, while the uterus, adrenals, prostate, lung, and lymph nodes contain very high levels (Albrechtsen, 1957). Activators have also been detected in milk, tears, saliva and urine. It had been considered that the tissue activators were stable and firmly bound to structural proteins requiring potassium thiocyanate for extraction (Astrup, 1947; Permin, 1950), but Albrechtsen (1958) was able to extract measurable amounts of activator from tissues by using physiological saline, suggesting that at least a part of the available activator was less firmly bound.

Fibrinolytic activity in most normal human tissues, as assessed by a histological autograph preparation technique (Todd, 1958), is directly related to their vascularity, and particularly their content of veins and

venules (Todd, 1964). It is not yet clear if the fibrinolytic activity observed by this technique represents the total tissue content of plasminogen activator, and the relationship between the activity observed by Todd, tissue activator as assessed by thiocyanate extraction methods and blood activator, has not yet been clarified.

Tissue activator from pig hearts has recently been isolated and purified (Bachman et al., 1964) but the preparation was still heterogeneous as assessed by electrophoresis and ultracentrifugation. Astrup and Kok (1965), were also able to prepare an activator from the ovaries of pregnant hogs, and this had a molecular weight of 58,000 and showed only two bands on starch gel electrophoresis. These preparations both showed certain physical and biological similarities to urokinase. Further biochemical investigation and purification will be of considerable interest.

Tissue activators may make a significant contribution to circulating blood activator (Maki et al., 1965) and be important in regulating the development of reparative connective tissue (Astrup, 1966).

Blood Activators.

Spontaneous fibrinolytic activity is a property of normal blood and the temperature labile factor responsible for this is a plasminogen activator. Activity is increased after a variety of physiological or pharmacological stimuli such as exercise or adrenalin (Biggs et al., 1947; Fearnley, 1952). Flute (1960) was able to separate electrophoretically a fraction of normal plasma which had similar properties to blood activator, but little else is known about its physico-chemical properties.

Todd (1959), Warren (1963) and Kwaan et al. (1956a), all suggested that one site of production of plasma plasminogen activator was probably the endothelium of capillaries, venules and veins, and that both vaso active changes and anoxia are related to its release (Holemans et al., 1965). It is not yet clear whether blood activator is a form of tissue activator similar to that extracted by Albrechtsen (1958) from saline washes of tissues as differences in stability to physical conditions and chemicals appear to exist between these forms of plasminogen activators.

There is some evidence that there may be a form of physiological control of the release of circulating plasminogen activator by neurogenic or hormonal mechanisms (Kwaan and McFadzean, 1956a, 1957; Schneck and Von Kaulla, 1961; Benetato et al., 1964). It is possible that a specific hypothalamic area is involved in this process. Other controlling mechanisms for circulating blood activator are poorly understood. Physiological stress such as exercise, pharmacological stimuli (adrenalin, nicotinic acid), will increase the levels of blood activator, while pregnancy decreases activity. There is now some evidence that specific organs may contribute to blood activator levels, particularly the kidney (Buluk and Furman, 1962; Holemans et al., 1967) and uterus (MacKay et al., 1967). However, the evidence regarding the release from the kidney is still controversial as Kucinski et al. (1968) were not able to demonstrate any difference in fibrinolytic activity between renal vein and inferior vena cavae blood. Activator is rapidly removed from the circulation apparently by the liver (Fletcher et al., 1964), or kidney (Celander and Guest, 1960; Holemans et al., 1966). The 50% in vivo clearance rate of plasminogen activator is

only 13 ± 5 minutes (Fletcher et al., 1964; Bachmann, 1966).

The mechanism by which plasminogen activator produces thrombolysis has been a subject of extensive study, as it is known that fibrin dissolution can occur without the appearance of free plasmin activity in the circulating blood. At least three theories have been advanced.

The first to be devised was that of Sherry et al. (1959).

Plasminogen, which is normally in close association with fibrinogen, is trapped in the interstices of blood clots during their formation. Plasminogen activator penetrates into the clot and is actively adsorbed onto the fibrin (Sherry, 1954). Within the clot, the entrapped plasminogen is converted to plasmin with consequent local, but not systemic lysis. The function of circulating anti-plasmin in this situation is to neutralize any free plasmin that should arise.

Another theory has been advanced by Ambrus et al. (1960). He proposed that small increases in the circulating levels of plasminogen activator produce plasmin which can subsequently be bound by antiplasmin. Upon contact with a clot, the plasmin-antiplasmin complex releases plasmin and this is transferred to fibrin, a substrate for which it has great affinity. In this scheme, circulating plasmin-antiplasmin serves as a reservoir of available plasmin for any substrate that has a greater affinity for plasmin than antiplasmin.

Wolf (1968) recently proposed a theory for the physiological action of fibrinolysis, similar to that earlier suggested by Olesen (1965). It was postulated that plasminogen activator and anti-activator exist as an easily dissociable complex, and this complex dissociates during

the diffusion of plasma proteins through the clot. Antiplasmins and anti-activators penetrate the clot less rapidly due to their large and/or asymmetric molecules. Thus by diffusion and dissociation, plasmin is formed internally in the clot, with subsequent clot lysis.

It is not proposed to discuss the pros and cons of these three theories as further investigation is required before definitive conclusions can be reached. Each, however, presupposes a key role for plasminogen activator in the physiological control of fibrin lysis.

Urokinase.

This physiological activator is present in normal urine, and can be purified (Ploug and Kjeldgaard, 1957; Fletcher et al., 1965) although contamination with thromboplastins has been a problem. It is now being utilized in thrombolytic studies on a small scale. Its physiological function could be to maintain patency of the urinary tract. It cannot be detected in peripheral venous or renal venous blood and is immunologically distinct from plasma activator, milk activator or tissue activators derived from the adrenals (Kucinski et al., 1968).

(ii) Non-Physiological Activators.

Streptokinase was identified by 1933 by Tillet and Garner, and has subsequently been purified for use in thrombolytic therapy. Initial material available was pyrogenic, but subsequent purification has reduced the incidence of such reactions. It is probable that streptokinase acts directly on plasminogen and/or plasmin converting it to an activator, at least in human plasma (Alkjaersig, 1964). Other non specific substances which

may cause plasminogen activation, at least in vitro, include peptones (Astrup and Olesen, 1957); urea (Von Kaulla and Smith, 1961); heparin (Halse, 1960); and protamine (Von Kaulla, 1952).

Other activators have been identified such as those derived from liquid cultures of Aspergillus oryzae (Stefanini et al., 1959), and from cell free filtrates of staphylococci (Lewis and Ferguson, 1951).

(d) Inhibitors of Fibrinolysis.

These are substances of endogenous or exogenous origin that will inhibit the action of plasmin or plasminogen activators. The in vivo parameters of the inhibitor system are less clearly defined mainly due to technical problems in the purification of reagents.

(i) Physiological inhibitors.

Antiactivators.

The separate existence of this type of inhibitor has not been proved as methods used for its measurement have not always distinguished it from antiplasmins. Some evidence exists for its presence (Lewis and Ferguson, 1951a; Kwaan et al., 1959; Flute, 1960; McNicol et al., 1963), but on the other hand Mann et al. (1966) could not demonstrate any discrete antiactivator activity in electrophoretically separated antiplasmin free human serum fractions. Further clarification is necessary.

Antiplasmins.

There are probably at least two antiplasmins in serum (Norman and Hill, 1958), one of which, an α_2 globulin, reacts rapidly as a

competitive inhibitor of plasmin, while the other in the α_1 fraction reacts slowly to form an inactive complex. Another antiplasmin may exist in the gamma fraction (Moriau et al., 1964). Platelets also possess antiplasmin activity (Johnson and Schneider, 1953), and these may be of importance in in vivo resistance to clot lysis. Normal plasma contains a great excess of plasmin inhibitors, presumably to prevent excessive fibrinolysis.

Calcium.

The mechanism of inhibition by calcium is not fully understood. It is possible that calcium participates in the formation of an antiactivator and potentiates the action of antiplasmins (Bruce, 1964). It is of interest that calcium appears to have different effects in the coagulation and fibrinolytic systems.

(ii) Non-Physiological Inhibitors.

Trypsin Inhibitors.

Both soybean trypsin inhibitor and pancreatic trypsin inhibitor have antiplasmin action and are used in research.

Chemical Inhibitors.

(a) E-aminocaproic acid (E.A.C.A.).

This is a potent, synthetic, competitive inhibitor of plasminogen activator (Ablondi et al., 1959), first investigated by Okamoto (1954). At very high concentrations it is a non-competitive inhibitor of plasma. Its efficacy in selected disorders of fibrinolysis has been well proved (McNicol and Douglas, 1964).

(b) Trasylo1.

This is a specific proteolytic inhibitor, extracted from bovine lungs, primarily inactivating plasmin but also preventing the activation of plasminogen. It also has an anti-thromboplastic activity (Amris, 1965; Nordstrom et al., 1967). Experience with its use in clinical conditions is growing (Dubber et al., 1965).

(c) Aminomethyl Cyclohexane Carboxylic Acid (A.M.C.H.A.).

This is a competitive inhibitor of plasminogen activator at low doses and a non-competitive inhibitor of plasmin at high doses. It has about twice the potency of E.A.C.A. but experience with its use is limited (Dubber et al., 1965).

(d) p-aminomethylbenzoic acid (P.A.M.B.A.).

Investigated by Okamoto and Okamoto (1962), and Lohmann et al. (1964). It is similar in action to A.M.C.H.A.

(e) Fibrin/Fibrinogen Degradation Products. (F.D.P.)

The importance of these split products of fibrinogen or fibrin in syndromes of pathological haemostasis has only recently been recognized. A full description of the spectrum and origin of these products is included in Section 1:3.

F.D.P. are derived by the proteolytic digestion of fibrin or fibrinogen by plasmin. Full digestion results in the formation of three classes of plasmin-resistant fragments. These include a fragment of

molecular weight 88,000 (the D fragment), another of molecular weight 30,000 (the E fragment), and a heterogeneous collection of low molecular weight fragments (Nussenzweig and Seligmann, 1960). Once formed in vivo the products have a half life of 9.5 hours (Fletcher et al., 1962), but their mode of excretion or further degradation is not known. The D fragment has antithrombic activity (Niewiarowski and Kowalski, 1958; Alkjaersig et al., 1962) as well as other effects on thromboplastin generation and platelet adhesiveness.

F.D.P. can be demonstrated by an immunological technique in most normal sera (Das et al., 1967).

CHAPTER III.

THE PHYSIO-PATHOLOGICAL CONCEPTS OF COAGULATION AND FIBRINOLYSIS

Introduction.

Both coagulation and fibrinolysis are intimately concerned with fibrinogen and fibrin. Recent research work has considerably clarified our knowledge of the proteolysis of these proteins by thrombin or plasmin; and as an understanding of these reactions is important in the interpretation of both physiological and pathological haemostatic changes, these subjects will be outlined in further detail before considering the postulated relationships between fibrin formation and lysis.

1. The Proteolysis of Fibrinogen by Thrombin.

The final step in the blood coagulation sequence is a complex but orderly reaction and has been studied extensively (Laki and Mommaerts, 1945; Lorand, 1951; Bettelheim and Bailey, 1952; Blombäck et al., 1962; Laki and Gladner, 1964). This process is known to occur in four main phases.

Firstly, thrombin, a highly specific proteolytic enzyme, acts on the fibrinogen molecule splitting it at arginyl-glycine linkages (Scheraga, 1961) into one major and several minor fragments. The minor fragments have been shown to be acidic polypeptides, and are named Peptides A and B (Bettelheim and Bailey, 1952). The molecular weights of these lie between 1,000 and 2,500 (Gladner et al., 1959). This initial phase can also be

subdivided, as thrombin releases Peptide A much more rapidly than Peptide B, and this alone is sufficient to bring about coagulation (Lorand, 1965), as Fibrinopeptide B only appears after appreciable amounts of fibrin have been formed. Secondly, fibrin monomer units rapidly polymerise and form a large hydrogen-bonded aggregate that becomes insoluble and is the definitive fibrin polymer. Thirdly, activation of fibrin stabilizing factor (F.S.F.) by thrombin takes place (Lorand and Konishi, 1962) in the presence of calcium ions. Lastly, bonding of the fibrin polymer takes place under the influence of the activated fibrin stabilizing factor.

These phases may be summarised as follows :-

1. Fibrinogen $\xrightarrow{\text{thrombin}}$ fibrin monomer and fibrinopeptides A + B.
2. $n(\text{fibrin monomers}) \xrightarrow{\text{Ca}^{++}}$ fibrin polymer.
3. $\text{F.S.F.} \xrightarrow[\text{Ca}^{++}]{\text{thrombin}}$ activated F.S.F. (F.S.F.*).
4. Fibrin polymer $\xrightarrow[\text{Ca}^{++}]{\text{F.S.F.*}}$ stabilised polymer.

Phase 1.

The first phase of this system has attracted great interest. For some time there was uncertainty as to the number of peptides released by the action of thrombin in its limited proteolysis of fibrinogen but further study, in a wide variety of different animal species (Blombäck, 1966) have demonstrated that two types of peptides, A and B, with few exceptions, are released. On chromatography two other peptides have been identified, peptides AP and Y, but these are regarded as being analogues of the A peptide, perhaps produced by the degradation of fibrinogen or fibrinopeptide either

in vivo or in vitro (Blombäck et al., 1966). Another analogue of the A and B fibrinopeptides is the E peptide fraction found in bovine clot supernatants and these derivatives have subsequently been shown to be produced probably during isolation (Pirkle et al., 1966). The amino acids sequences of these peptides have been elucidated (Blombäck et al., 1959; Folk et al., 1959; Sjöguist et al., 1960) and more than twenty different animal species have been worked out in the laboratory (Blombäck, 1967). For human peptide A and peptide B, these are :-

Peptide A.

H-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-OH

Peptide B.

$$\begin{array}{cccccccccccccccc} & & \text{NH}_2 & & \text{NH}_2 & & & & & & & & & & & & & & \\ & & | & & | & & & & & & & & & & & & & & \\ \text{Pyr-Gly-Val-Asp-Asp-Asp-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH} \end{array}$$

The role of these peptides in physiological situations is an exciting new field for investigation, for not only may they act as homeostatic inhibitors in the clotting mechanism (Silver and Murray, 1966), but their release may be important in the control of the haemodynamics of the microcirculation. It has been shown that they potentiate the action of such substances as bradykinin and histamine (Osbahe et al., 1964, 1967) and have physiological effects when infused in minute doses on the blood flow of both the heart and lungs (Bayley et al., 1967).

Phase 2.

Thrombin attacks the fibrinogen molecule at a specific site,

considered to be the N-terminal end of the molecule (Blombäck, 1967). The resultant fibrin monomer can remain soluble in the blood stream but can also polymerise with unaltered fibrinogen in conjunction with F.S.F. to form a complex, soluble at high concentrations of fibrinogen at 37°C but forming a cryoprecipitate at 4°C (Sasaki et al., 1966) named cryoprofibrin by Shainoff and Page (1962). However, in this second stage of coagulation, the removal of the negatively charged peptide particles from the fibrinogen molecule usually appears to allow fibrin monomers to aggregate and in the presence of calcium, to form fibrin polymer (Lorand, 1965). Loss of the peptides may also open up hydrogen binding sites on the fibrinogen molecule allowing inter molecular binding (Scheraga, 1961). The fibrin units align themselves longitudinally, but in a staggered arrangement, giving rise to a periodicity characteristic for fibrin when viewed by electron microscopy (Hall and Slayter, 1959; Bang, 1964). This formed fibrin polymer is not, however, strong enough to fulfil its physiological functions, and stabilisation of the molecule can only be achieved when crosslinks are formed.

Phases 3 and 4.

In plasma this occurs in two stages; the initial action is of thrombin activating fibrin stabilising factor (Blombäck and Doolittle, 1963), followed by the activated F.S.F., apparently through a transpeptidating mechanism (Lorand, 1965), firmly linking longitudinally arranged fibrin polymers together, thus completing the normal fibrinogen to fibrin transformation. This bonded fibrin is also now less susceptible to lysis (Tyler and Lack, 1965).

This carefully elucidated sequence of events has clarified an

important aspect of coagulation, and aids in the explanation not only of certain clinical occurring coagulation anomalies but also many of the differences occurring in physical measurements between fibrinogen and fibrin as assessed by immunoelectrophoresis, electron-microscopy and protein finger printing (Blombäck, 1967).

2. The Proteolysis of Fibrinogen by Plasmin.

The enzyme plasmin degrades fibrinogen (molecular weight 340,000) sequentially into at least three classes of fragments. These comprise (a) a large molecular weight derivative (265,000) which although clottable by thrombin has an altered molecular configuration and clots slowly and abnormally (Fletcher et al., 1966); (b) intermediate fibrinogen derivatives ranging in molecular weight between 200,000 and 100,000, these products demonstrating varying degrees of thrombin clottability, and (c) the final plasmin-resistant derivatives consisting of a fragment of 88,000 molecular weight (the D fragment named after its fibrinogen antigenic determinant), a fragment of 30,000 molecular weight (the E fragment), and a variety of low molecular weight non-antigenic fragments. These final groups of plasmin resistant molecules have been extensively studied (Nussenzweig and Seligmann, 1960; Alkjaersig et al., 1962), chromatographically (Nussenzweig et al., 1961), electrophoretically (Fletcher et al., 1966) and quantitatively (Ferreira and Murat, 1963), and are usually referred to as fibrinogen/fibrin degradation products (F.D.P.).

Defective fibrin polymerisation is a term used to describe the coagulation anomaly characterised by the appearance in the circulation of

these plasmin derived fragments (Fletcher et al., 1966) from either fibrin or fibrinogen. Two types of defective fibrin polymerisation are recognised:

(i) Proteolysis of fibrinogen initially produces fibrinogen derivatives which are still thrombin clottable although slowly and defectively. This slow clotting phenomena may be explained on the basis of a molecular alteration that shields thrombin susceptible sites from proteolysis, or by a concept that the altered molecule, although susceptible to normal thrombin action, polymerises poorly (Fletcher et al., 1966).

(ii) With the appearance of the non-clottable smaller molecular size (D and E) fibrinogen fragments and their bonding with fibrin monomer to form a complex, sufficient bonding sites are not available to link completely with other fibrin monomers. Polymerisation therefore is slow and defective with the formation of a morphologically inadequate clot (Bang et al., 1962) which also has a marked deficiency of mechanical strength (Hirsch et al., 1965).

It has now become apparent that the detection of abnormal fibrin polymerisation states is of considerable clinical importance. Using immunoelectrophoretic and immunodiffusion techniques, Fisher et al. (1967) were able to identify, in patients with very mild proteolysis, the appearance of large early fibrinogen derivatives before the development of other biochemical abnormalities. Thus biochemical recognition of defective fibrin polymerisation in its very earliest stages is now possible on a research basis. Much more work has been focussed on the plasmin resistant fragments (D and E) and their measurement has been of considerable clinical

use in the diagnosis of defective fibrin polymerisation (Ferreira and Murat, 1963; Nilehn and Nilsson, 1964; Merskey et al., 1966), associated with disseminated intravascular coagulation (McKay, 1965).

The liberation of split products of fibrinogen may also affect other haemostatic factors, besides fibrin polymerisation. They may have an antithrombic effect (Niewiarowski and Kowalski, 1958; Triantaphyllopoulos and Triantaphyllopoulos, 1966), an anti-thromboplastic effect (Niewiarowski et al., 1959; Nilehn, 1967), and also affect the adhesiveness of platelets (Kowalski et al., 1963).

In this thesis considerable attention has been directed to the changes occurring in both physiological and pathological situations, of the plasmin resistant moieties, i.e. the D and E fragments of fibrin, usually referred to as fibrin/fibrinogen degradation products (F.D.P.).

The proteolysis of fibrinogen by plasmin or thrombin may be summarised in Fig. 1:3.



THE PROTEOLYSIS OF FIBRINOGEN BY THROMBIN AND PLASMIN

Fig. 1:3.

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Fig. 1:3. THE PROTEOLYSIS OF FIBRINOGEN BY THROMBIN AND PLASMIN

Fig. 1:3.

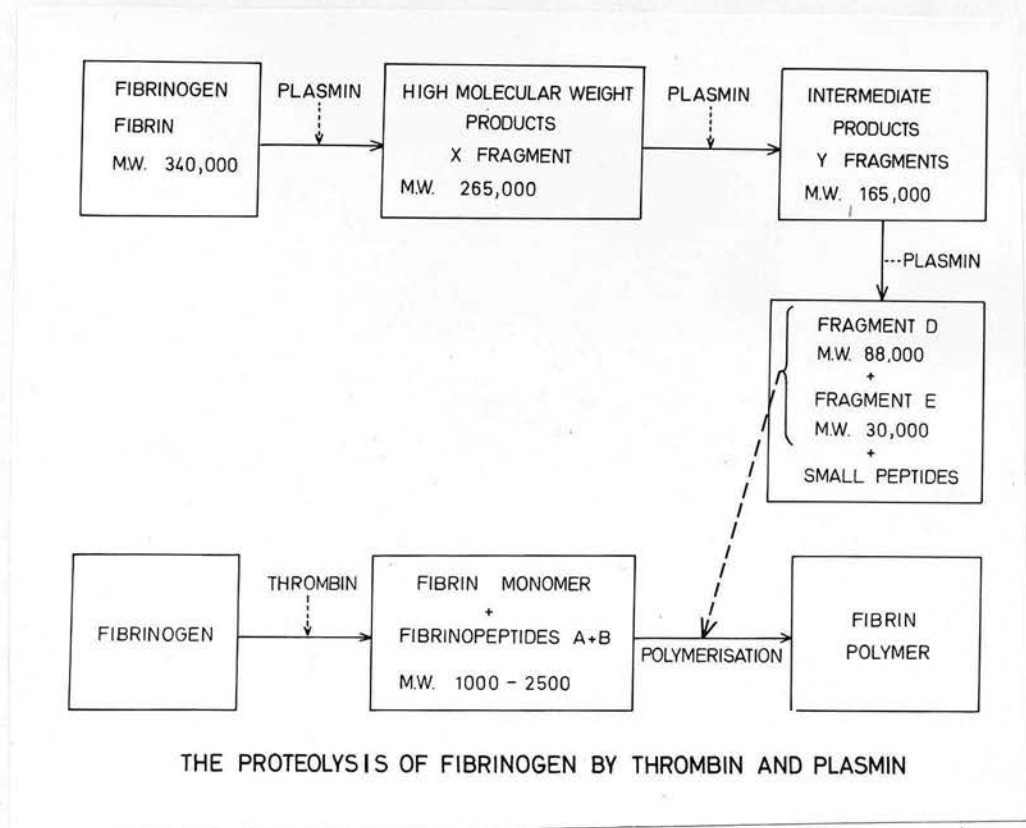


Fig. 1:3.

3. The Coagulation-Fibrinolytic Equilibrium.

It is axiomatic that continuous coagulation and fibrinolysis must proceed in vivo at a balanced speed, if normal tissue maintenance and structural integrity is to be preserved. Whether normal vascular patency also depends on a similar process is, however, debatable. It is evident that some such mechanism must be present to maintain blood fluidity but clear definition is still evasive despite much research.

Astrup (1956) summarised and extended the views already put forward by Nolf (1908) and Copley (1954), that the two systems were in a continuous dynamic balance of fibrin deposition and removal. In its fullest concept it was envisaged that fibrin deposition was a continuous in vivo process, in which the endothelial lining of blood vessels were coated with a thin layer of fibrin continually being removed by the action of the fibrinolytic system, thus ensuring the patency of the blood vascular system. Disturbances of this fine balance would result in excessive bleeding on one hand, and a thrombotic tendency on the other. There was considerable evidence to support such a view and the hypothesis has subsequently stimulated much research. The concept was strengthened by knowledge of the known very short half lives of the coagulation factors (Bowie et al. - review - 1967), the demonstration of a layer of fibrin on the walls of some blood vessels (Copley, 1954; Woolf, 1961), and the finding of active fibrinolysis in normal blood. Contrary to the hypothesis was the finding of a normal turnover of clotting factors in patients treated with anticoagulants (Lewis et al., 1961), the lack of intravascular thrombi formation in subjects on fibrinolytic inhibitors (Gajewski and Alexander, 1963; Lewis, 1963), the

absence of continuous spontaneous bleeding and the normal turnover of clotting factors in patients with hereditary clotting defects (Adelson et al., 1961; Rausen et al., 1961; Hart, 1965), and the fact that fibrin cannot be demonstrated on normal endothelium (Woolf and Crawford, 1960).

The two sides of this convenient hypothesis have been summarised adequately in 1961 and 1965 by P.J. Hjort. Further evidence both for and against the hypothesis is still becoming available (Das et al., 1967; Regoesczi, 1967; Regoesczi and Walton, 1967) and it is likely that the theory will become considerably modified. Many authorities now agree that the mechanism is essentially designed for local action and that a continuous dynamic balance, as originally envisaged is less likely (Astrup, 1967). The role of the reticulo endothelial system in the removal of intermediate derivatives of coagulation is probably also of considerable importance (Spaet, 1966).

It is still possible however, that in vivo thrombin activity may occur continuously. Adelson (1965) and Laki (1968) suggest that partial thrombin polymerisation of fibrinogen with release of polypeptides may occur normally in the circulation. Direct proof of this is lacking but demonstration of these peptides in normal subjects would be of considerable interest. Shainoff and Page (1964) have also proposed that monomers of fibrinogen can also occur in the blood stream, and these may be linked with fibrinogen molecules in soluble conjugates which, under certain conditions, may precipitate as fibrin.

Very little is known as to the overall controlling mechanisms of the coagulation and fibrinolytic systems. Although it is evident that hormonal

changes, as in pregnancy or in exogenous oestrogen or progesterone treatment, probably influence the absolute levels of both clotting and fibrinolytic factors, the physiological role of these and other hormones is unknown (Thompson and Poller, 1965; Brakman et al., 1967). There is some evidence that neural pathways may, in some way, modify the systems (Kudrjashov, 1959; Markosian and Yakounine, 1961; Gunn and Hampton, 1967; Correll, 1968), but little accurate information is available.

4. Disseminated Intravascular Coagulation. (D.I.C.)

The concept of disseminated intravascular coagulation as an intermediary process in many separate disease processes, has been developed in recent years by the experimental work and clinical observations of both McKay (1965) and Hardaway (1966). It may be regarded as a logical extension of the postulated theory of a physiological balance between coagulation and fibrinolysis, with a marked shift in equilibrium resulting in the pathological formation of fibrin and a subsequent activation of fibrinolysis. Its full description, however, includes more than the simple formation of a thrombus and McKay and Muller-Berghaus (1967) have defined it adequately as "a biological process involving many chemical substances and physiological responses. It begins with the entry of a pro-coagulant material or activity into the circulating blood; progresses to the stage of platelet aggregation and fibrin formation which may or may not result in thrombosis of capillaries, arterioles and venules of various organs; it is associated with activation of the fibrinolytic system, with dissolution of fibrinogen and fibrin and the

release of fibrin split products into the plasma; and is not complete until the haemostatic mechanism and vasomotor apparatus have returned to normal, and the last significant amount of fibrin split product has been cleared from the blood."

The degree of D.I.C. occurring may vary greatly. Although it is possible that minor degrees of D.I.C. may occur in response to physiological stresses, the syndrome has been most commonly recognised in pathological states associated with haemolysis, release of tissue thromboplastins, bacterial endotoxins, proteolytic enzymes, anoxia and anoxemia and endothelial damage. In some patients, intravascular coagulation may be dominant, while in others, fibrinolysis overshadows the initiating coagulation process. The laboratory findings usually include diminution of clotting factor levels, and evidence of activation of fibrinolysis. The demonstration of fibrin split products is strong confirmatory evidence. The degree of change found in these parameters may vary widely, and this may depend to some extent on the severity of the disease process and the clinical stage at which blood is tested.

It is possible that the pathological sequelae of D.I.C. occur only when the total capacity of the body's reticulo endothelial and fibrinolytic systems are unable to cope rapidly with the clearance of coagulation intermediates and fibrin. Such would appear to be the case in obstetric emergencies, e.g. amniotic fluid embolism and abruptio placentae, and perhaps also in incompatible blood transfusion reactions. In other situations, the initiating mechanism is not always so clearly defined, but may be related to anoxia, intravascular haemolysis and the local release of

tissue thromboplastins.

Well documented reports of D.I.C. have become available in such varied conditions as carcinomatosis, acute leukaemias, overwhelming infections, thrombotic thrombocytopenic purpura, severe shock, following extra corporeal circulation, snake venom poisoning and many others (Sherry, 1966). It is likely that identification of D.I.C. will increase as methods of recognition, such as F.D.P. assays, become generally available.

Although the concept clarifies many aspects of abnormal haemostasis, it is still necessary to avoid over interpretation of its incidence or importance as some of the experimental work on which the theory is based may not be necessarily applicable to disease in man. Nevertheless, it is providing a significant focus for further research into the causes of haemorrhage and thrombosis and will almost certainly provide new advances in present therapeutic regimes.

5. Shwartzman Reactions.

The classical generalised Shwartzman Reaction is produced in young rabbits by the intravenous injection of Gram-negative endotoxin spaced 24 hours apart. After the first injection a few fibrin thrombi may be found in liver, lungs and spleen. After the second injection fibrin thrombi can be detected in glomerular capillaries and splenic sinusoids, resulting in bilateral renal necrosis and splenic haemorrhage and necrosis (Thomas and Good, 1952).

Thrombin (Lee, 1962) or antigen-antibody reactions (Lee, 1963) can substitute for the second "provoking" injection. The first ("preparing")

injection can be replaced by pregnancy (Apitz, 1935), epsilon-amino caproic acid (Lee, 1962), steroids (Thomas and Good, 1952), and "Thorotrast" (Good and Thomas, 1952). The reaction is regarded as being caused by acute intravascular coagulation, and heparin protects against the reaction (Good and Thomas, 1952).

This classical reaction can occur in man as evidenced by therapeutic misadventures whereby repeated doses of typhoid vaccines were administered for other purposes. At least three patients died with severe liver and renal damage; the latter disorder reported as acute tubular necrosis (Lewis, 1941; Urbach et al., 1944; Love and Driscoll, 1945). However, it is now clear that the complete Shwartzman reaction is rare in human diseases, but modified forms do appear in certain clinical conditions associated with disseminated intravascular coagulation. The most common examples are those associated with complications of pregnancy, such as premature placental separation and septic abortions.

This concept, as applied to human diseases, has created considerable scope for research, to which must be added areas of the work included in this thesis.

6. Fibrinolysis and Occlusive Vascular Disease.

If fibrinolysis is the physiological antithesis of coagulation, its study should be relevant to the problems of occlusive vascular disease. Rokitanski (1842) suggested that deposits of fibrin on the arterial wall caused atherosclerosis, and Astrup (1956) linked this theory with the dynamic equilibrium hypothesis of haemostasis and also to that of Duguid (1949, 1955), who had revived the idea of fibrin being an aetiological factor

in arterial degenerative disorders. The factors affecting the resolution of fibrin could therefore be of some significance and considerable effort has been undertaken to assess the relevance of depressed fibrinolytic activity to occlusive vascular disease. Many of these studies have proved unrewarding and controversial (Hume, 1958; Nestel, 1959; Lackner and Merskey et al., 1960; Ogston, 1962; Naimi et al., 1963; McKay and Hume, 1964) although evidence has accumulated to suggest that men with arteriosclerosis of the lower limbs have a greater evidence of defective fibrinolysis than controls (Fearnley et al., 1964).

The results in coronary heart disease have been discordant although the most recent work by Chakrabarti et al. (1968) does suggest that significantly depressed fibrinolytic activity is found in patients with coronary artery disease up to the age of 60. If the results of this study can be substantiated, prophylactic treatment of selected patients with drugs (or onions - Menon et al., 1968) that enhance fibrinolysis might become a clinical reality. However, much more systematic study is required using more specific techniques, before definitive conclusions can be drawn on the postulated relationship between decreased fibrinolytic activity and occlusive vascular disease.

SECTION 2.

AIMS OF PROJECT AND INTRODUCTORY REVIEW.

AIMS OF PROJECT.

This study has three main objectives in view.

1. The development, refinement and standardization of a wide range of analytical and physiological assays, to a level suitable for research into physiological and pathological states.

This work is described in Section 3.

SECTION 2.

AIMS OF PROJECT AND INTRODUCTORY REVIEW.

This work is described in Sections 4 and 5.

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2. The definition of the normal values for parameters of the various physiological systems as measured in this laboratory, as a guide to the interpretation of pathological variations occurring in the experimental conditions.

This work is described in Section 5.

In this section, it is appropriate to review briefly the concepts of physiological measurement as originally developed in this laboratory. The present will be referred to and elaborated in the discussion associated with the physiological studies.

SECTION 2.

AIMS OF PROJECT AND INTRODUCTORY REVIEW

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This thesis has three main objectives in view.

1. The development, refinement and standardisation of a wide range of coagulation and fibrinolytic assays, to a level suitable for research into physiological and pathological states.

This work is described in Section 3.

2. The further investigation and extension of previous research work on the "fibrinolytic reactivity" to various stimuli, in both normal and pregnant subjects.

This work is described in Sections 4 and 5.

3. The collection of the normal values for pregnancy of the various fibrinolytic parameters as performed in this laboratory, as a guide to the further interpretation of pathological variations occurring in the haemostatic mechanism.

This work is described in Section 5.

At this juncture, it is appropriate to review briefly the concept of "fibrinolytic reactivity" as originally developed in this laboratory. This concept will be referred to and elaborated in the discussions associated with each subsequent chapter.

INTRODUCTORY REVIEW.

The concept of the reactivity of the fibrinolytic system being of relevance in physio-pathological situations is not new. Fearnley et al. (1951) first attempted to investigate this hypothesis using injections of adrenalin, but met with little success, probably as a result of the inadequacies of methodology at that time. It was not until 1966 that work in this laboratory defined the concept of fibrinolytic reactivity more clearly (Cash, 1966). Using exercise and adrenalin infusions, and measuring changes in euglobulin lytic activity with carefully controlled techniques, it was shown that fibrinolytic response in an individual was a reproducible phenomena, and that responses in an apparently healthy population followed a normal distribution, with both high, moderate and low responders. It was also demonstrated that the response of each subject was similar when different stress methods were used, i.e. a low responder would remain in this category when stimulated with either moderate or severe exercise, or adrenalin (Cash and Allan, 1967; Cash and Woodfield, 1967).

From this work, it was postulated that the poor fibrinolytic responder might be more susceptible to conditions in which either focal or disseminated intravascular coagulation could occur. The ability to rapidly generate plasminogen activator to stress procedures could thus conceivably be an aetiological factor in the pathogenesis of such disorders as atherosclerosis, irreversible shock and thrombosis, conditions in which a defective fibrinolytic system has been suggested as playing some part.

The evidence for a fibrinolytic response being of significance

in patho-physiological events was, however, meagre. If it could be shown that other physiological stimuli could affect the fibrinolytic response, or that patients with vascular pathology also exhibited impairment of response, this would be at least some proof of the hypothesis. Both these facets were subsequently studied and it was discovered that the fibrinolytic response could be modified by mental stress (Cash and Allan, 1967) and was lowered in diabetics (Cash and McGill, 1968).

It was from these studies that the present work was developed. Assuming the coagulation and fibrinolytic systems are dynamically balanced, it was suggested that the poor fibrinolytic responder might not necessarily be at risk if the coagulation system was compensating by forming less fibrin. The reactivity of the coagulation system to exercise stresses and prolonged adrenalin infusions were therefore investigated and the results have been documented in this thesis in Sections 4:1,2. Also, in view of the modification of the fibrinolytic response to mental stress, and the accumulated evidence that the neural system could be involved in the control of coagulation and fibrinolysis, the effect of β -receptor adrenergic blockade on these systems, were also studied.

It was also possible that the fibrinolytic response could be relevant in clinical conditions associated with episodes of disseminated intravascular coagulation. In pregnancy, the incidence of this complication is relatively high, and an investigation was therefore undertaken of the fibrinolytic reactivity of pregnant women to exercise stress, this being documented in Section 5:2. Concurrently, the normal values of fibrinolytic parameters at the varying stages of gestation were assessed (Section 5:1).

In a further study (Section 5:3), the reaction of the fibrinolytic system to an operative surgical stress, both in the pregnant and non-pregnant state, was intensively investigated. Such a study extended even further than the concepts of the fibrinolytic response, and it was considered that the results could be of relevance to both clinical bleeding and thrombotic disorders.

Finally, patients in whom the syndrome of disseminated intravascular coagulation had occurred were carefully investigated using all available coagulation and fibrinolytic assay techniques (Section 5:4). This study assessed, in a more general sense, the reactions of both the coagulation and fibrinolytic systems to a pathological stress.

In these studies, use has extensively been made of a recent developed technique for the measurement of fibrinogen/fibrin degradation products (Merskey et al., 1966). This assay undoubtedly introduces a new approach to both the physiology and pathology of the haemostatic mechanism but also raises fundamental problems in our previously held conceptions of the coagulation and fibrinolytic systems.

A fuller discussion of the various separate but interrelated subjects has been included with each chapter, along with reviews of the relevant literature.

SECTION 3.

LABORATORY METHODOLOGY



CHAPTER I.

MATERIALS.

A. Equipment.

1. Balance.

Oertling R.20. L. Oertling Ltd., Orpington, Kent.

2. Centrifuges.

(a) B.T.L. Bench centrifuge, C 6/0075.

Baird and Tatlock Ltd., Chadwell Heath, Essex.

(b) M.S.E. Mistral 4L, refrigerated centrifuge.

Measuring and Scientific Equipment Ltd., London,
England.

(c) M.S.E. Minor bench centrifuge.

Measuring and Scientific Equipment Ltd., London,
England.

3. pH Meter.

Beckman Zeromatic, Beckman Instruments Ltd., Glenrothes,
Scotland; with glass electrode supplied by Activion Glass Ltd.,
Kinglassie, Fife, Scotland.

- (c) Pyrex Glass Centrifuge Tubes - 10, 50 and 100 ml.
- (d) Glass Pipettes 0.1 - 25.0 ml. Trademark "E-Mil",
H.J. Eliot Ltd., Glamorgan.
- (e) Plastic tubes, made of clear polystyrene and produced by
Teklab Laboratories, Hockley, Essex.

9. Syringes and Needles.

- (a) Disposable plastic syringes (2-50 ml.). Plastipak,
produced by Becton, Dickinson & Co., Drogheda, Ireland.
- (b) Glass syringes (50 ml.). Sterilised in a hot air oven in
special packs.
- (c) Needles: 20G x $1\frac{1}{2}$ sterile disposable; Becton, Dickinson & Co.
21G x $1\frac{1}{2}$ sterile disposable; Gillette Scimitar,
Middlesex, England.

10. Microtiter set.

Produced by Cooke Engineering Company, 735 North Saint Asaph Street,
Alexandria, Virginia 22314, U.S.A.

The Microtiter Kit consisted of :-

- (a) Lucite transparent plates, each containing 96 conical bottom wells.
- (b) Calibrated pipette droppers (delivery volume 0.025 ml.) made of
semi-transparent polypropylene with stainless steel droppers.
- (c) Microdiluters, made of stainless steel and calibrated to deliver
0.025 ml.
- (d) A multi-microdiluter gun, this instrument enabling rapid performance
of dilution processes.

4. Spectrophotometer.

Unicam S.P.600. Unicam Instruments Ltd., Cambridge, England.

5. Coagulometer.

Four channel automatic clot timers built by Depex, de Bilt, Holland, were used. Full details are included in Section 3:4. The electrode cleaner for the machine was built in the department.

6. Clot Lysis Recorder.

A twelve channel clot lysis recorder designed within the laboratory was used (Cash and Leask, 1967). The apparatus records in graphical form, the alterations occurring in optical density as lysis occurs in plasma clots.

7. Constant Infusion Pump.

Harvard two channel constant infusion pump with variable infusion speeds.

8. Glass and Plastic Ware.

(a) Clotting tubes (5" x $\frac{1}{2}$ "; 3" x $\frac{3}{8}$ "; 52.5mm x 11.5mm).

Tubes selected for use were produced by Samco, S. Murray & Co., Old Woking, Surrey. These were of plain glass and were not washed prior to use. They were siliconised as required, by the method outlined in the Appendix.

(b) Test tubes (5" x $\frac{1}{2}$ "; 5" x $\frac{3}{8}$ ")

Plain glass.

Washing procedure as per Appendix.

11. Glass Capillary Tubes.

Produced by British Drug Houses, Poole, England. Length 10 cms., internal diameter 0.1 cm.

12. Petri Dishes.

Supplied by Staynes Laboratories Ltd. Each dish had an internal diameter of 8.8 cms. and was disposable.

B. Reagents.

1. Fibrinogen.

(a) Human and Bovine fibrinogens.

A preparation supplied by Kabi Pharmaceuticals, Ealing, London, was used. The fibrinogen had a clottability of 90% and was prepared by the method of Blomback and Blomback (1956).

(i) For use in fibrin plate assay method.

7.5 gm. of fibrinogen was added to 500 ml. of tris buffer pH 7.8. The fibrinogen was dissolved at 37°C with gentle stirring, and then stored in small aliquots in plastic tubes at -40°C. For use, this 1.5 gm.% fibrinogen solution was diluted 1 in 10 in buffer.

(ii) Standard reference preparations for F.D.P. assay.

Fibrinogen was diluted to concentrations of 5, 2.5 and 1.25 mgm/ml. in buffer and stored in small aliquots at -40°C.

(b) Bovine fibrinogen.

As supplied by Diagnostic Reagents Ltd., Thame, Oxon, for

use in coagulation techniques. Batch Numbers F.N. 31 - 33 were used.

2. Thrombin.

(a) Bovine Thrombin.

Supplied by Parke Davis & Co., Detroit, Michigan, U.S.A., containing 5,000 N.I.H. units per vial. It was diluted to varying concentrations for different tests.

(i) Euglobulin lysis time assay.

A solution of 5 U/ml. in veronal acetate buffer (pH 7.4) was made, and small aliquots were stored in plastic tubes at -40°C .

(ii) Fibrin plate assay.

A solution of 50 U/ml. in tris buffer (pH 7.8) was made and small aliquots were stored in plastic tubes at -40°C .

(iii) Fibrinogen assay (Ellis and Stransky method)

A solution of 10 U/ml. was made in 0.9% saline, and to this was added an equal volume of 1.12 M solution of calcium chloride. Aliquots were stored in plastic tubes at -40°C .

(b) Human Thrombin.

Supplied by the Blood Products Laboratory, Lister Institute, Elstree, Herts (Batch Number 545D). This was used for coagulation techniques and was diluted to a concentration of 50 U/ml. in tris buffer (pH 7.4) and stored in small aliquots at -40°C . For use, it was diluted in buffer to give a clotting time on normal pooled plasma of between 12 and 15 seconds.

3. Thromboplastin.

A phenolised suspension of rabbit brain thromboplastin as supplied by Diagnostic Reagents Ltd. was used. Batch Numbers K.B. 60 - 62.

4. Arvin Snake Venom.

Supplied by Twyford Laboratories Ltd. in ampoules containing 0.9 ml. of specific activity 82 units/ml. Batch No. T.80.

5. Phospholipid.

Prepared according to Bell and Alton (1954), and supplied by Diagnostic Reagents Ltd. Batch Numbers R.B. 19 and 20.

6. Kaolin Platelet-Substitute Reagent.

Supplied by Diagnostic Reagents Ltd. Batch Numbers KC.14 - KC.22 were used and fuller description of this reagent is included in Section 3:2.

7. Purified Factor V.

Supplied by Diagnostic Reagents Ltd. Batch Numbers F.B. 23 - 25.

8. Russell's Viper Venom/Cephalin Mixture.

Supplied by Diagnostic Reagents Ltd. Batch Numbers R.C. 19 - 21.

9. Heparin B.P.

Evans Medical Ltd., Speke, Liverpool. Ampoules of 5,000 I.U./ml. were diluted in saline to a concentration of 20 U/ml. and stored at 4°C.

10. Urokinase.

The standard preparation used was produced by Leo Pharmaceuticals, Denmark. Vials containing 2,400 Plough Units were diluted to 3 units/ml. in tris buffer pH 7.8, and aliquots were stored at -40°C in plastic tubes.

11. Aluminium Hydroxide moist gel.

British Drug Houses, Poole, Dorset. For use 5 gms. of gel were suspended in 20 ml. of distilled water and stored at 4°C .

12. Celite 512.

Johns Manville & Co. Ltd., New York, U.S.A. 1.0 gm. was suspended in 10 ml. of 0.9% saline prior to use.

13. Molar Calcium Chloride.

British Drug Houses. This solution was diluted as required to 0.025 M and 0.05 M concentrations.

14. Coagulation Factor Deficient Substrate Plasmas.

(a) Factor VIII, IX and XII deficient plasmas.

These were collected from patients known to have severe coagulation factor deficiencies. Blood was spun at 3,200 r.p.m. at 4°C for 30 minutes, the plasma separated and stored in aliquots of 1.7 ml. in plastic tubes at -40°C . Plasma for Factor XII assays was obtained from Professor E. Loeliger, Leiden, Holland.

(b) Factor V deficient plasma.

This was artificially prepared as described in Section 3:2.

(c) Factor X assay substrate plasma.

Supplied by Diagnostic Reagents Ltd. Batch Numbers

C. 65 - 71.

15. Fibrinogen Antisera.

Rabbit anti-human fibrinogen antisera was obtained from Hoeschst Pharmaceuticals Ltd., Brentford, Middlesex. Batch Numbers A.C. 806 and 1202 M were used. The antisera was diluted 1:500 in 0.15 M citrate buffer (pH 6.4) containing sodium azide (1 mgm/ml.) and adsorbed horse serum 1:250 and stored in small aliquots at -40°C .

16. Trasylol.

Obtained from Farbein Fabriken Bayer A.G., Germany, in vials containing 25,000 K.I. Units in 5 ml.

17. Sheep Red Cells in Alsevers Solution.

Supplied by Burroughs Wellcome & Co., London. Stored at 4°C .

18. Buffers.

As in Appendix.

CHAPTER II.

COAGULATION TECHNIQUES.

For the investigation of blood coagulation most techniques were carried out at 37°C in a water bath or heating block. Dilutions of plasma as well as certain reagents were kept on melting ice in a water bath at 4°C .

1. Whole Blood Clotting Time. (W.B.C.T.)

The method was similar to that of Lee and White (1913) but as described by Macfarlane and Biggs (1962).

The technique was as follows :-

(a) Glass Clotting Time.

Three new, unwashed, non-rinsed $3'' \times \frac{1}{2}''$ test tubes were placed in a water bath at 37°C . Venous blood was obtained by careful venepuncture and minimal venous occlusion, using a plastic syringe with a needle of gauge size 20. A stopwatch was started as the blood entered the syringe; 1.0 ml. of blood was then transferred to each clotting tube. The tubes were left undisturbed for 4 minutes, then tilted gently at 30 second intervals until unequivocal solidification had occurred. The clotting times of the three tubes were recorded and the average of these tests was taken as the clotting time. The mean value of 15 estimations on normal subjects in the 20 - 30 year age range was 8.9 ± 1.6 minutes.

(b) Silicone Clotting Time.

The technique was identical to the above with the exception that siliconized tubes (I.C.I. M550 silicone in trichlorethylene), were used and these were left undisturbed after the addition of blood for 12 minutes. The mean value and standard deviation on 15 normal subjects of age range 20-30 years, was 19.6 ± 2.2 minutes.

2. Plasma Recalcification Time. (R.C.T.)

The method was as described by McDonald and Fullerton (1958.).

(a) Platelet Rich Plasma.

Venous blood collected by the usual method was spun at 1,000 r.p.m. (150g) at room temperature for exactly 5 minutes. The plasma was then removed using a siliconized pipette and tested immediately.

0.2 ml. of plasma was warmed to 37°C for exactly 2 minutes, either in triplicate $3" \times \frac{3}{8}"$ clotting tubes (for visual clotting times) or $52.5 \text{ mm} \times 11.5 \text{ mm}$ tubes (for coagulometer clotting times). 0.2 ml. of 0.025 M calcium chloride was then added and the clotting time recorded. The mean value for this test was 112 ± 14 seconds as measured in 20 normal subjects. The mean percentage error as calculated from 30 tests was 2.1%.

(b) Platelet Poor Plasma.

Venous blood, collected in the usual manner was spun at 3,450 r.p.m. (2,230g) for 20 minutes. The plasma was separated with a siliconized pipette and tested immediately. The procedure was otherwise as for (a).

The mean value for this test was 171 ± 46 seconds as measured in

50 normal subjects. The mean percentage error, as calculated from 50 tests was 2.4%.

3. Kaolin-Cephalin Clotting Time. (K.C.C.T.)

The test is sensitive to changes in the levels of clotting Factors I, II, V, VIII, IX, X, XI and XII, i.e. the intrinsic system of coagulation. The method adopted was similar to that described by Matchett and Ingram (1965). Surface activation of the plasma is supplied by kaolin, and a partial thromboplastin reagent is also added to increase the rate of reaction and reduce any effect of varying plasma platelet concentrations.

(a) Kaolin-platelet substitute reagent.

The partial thromboplastin reagent used was a commercial preparation also incorporating kaolin. (Kaolin-platelet substitute reagent - Diagnostic Reagents Ltd.).

This lyophilised product contains kaolin (2 mgm/ml.) and a platelet substitute (rabbit brain phospholipid prepared according to Bell and Alton, 1954), and is supplied in 10 ml. vials. In view of reports of instability of certain platelet substitutes available (Veltkamp, 1965), and the importance of the reagent to many of the assay techniques, the following experiments were performed.

(i) Stability after Reconstitution

The kaolin-cephalin clotting time was performed, at intervals of 1 hour (throughout a day) on freshly thawed aliquots of fresh frozen plasma, using kaolin-platelet substitute reagent stored at either 4°C or 37°C. The

results of experiments on three separate plasmas are summarised in Table 3:1. There is no significant alteration in the stability of the reagent over the time period studied (7 hours) between the reagent stored at 4°C or 37°C.

TABLE 3:1

THE STABILITY OF KAOLIN-PLATELET SUBSTITUTE REAGENT
ON STORAGE AT 37°C AND 4°C.

Hours	0	1	2	3	4	5	6	7	
Plasmas	K.C.C.T. in Seconds								
37°C	1	40.9	40.9	39.0	38.8	39.9	39.4	39.0	37.2
	2	39.0	39.0	38.8	39.0	38.2	39.2	39.4	38.4
	3	36.0	36.6	37.1	36.4	36.4	35.4	37.4	36.9
Mean	38.6	38.8	38.3	38.1	38.2	38.0	38.6	37.5	
4°C	1	40.2	39.5	39.4	39.6	38.9	39.5	38.9	39.4
	2	39.4	39.5	39.6	38.8	38.5	39.6	38.4	38.8
	3	36.5	36.5	36.5	37.0	36.6	36.7	36.4	37.8
Mean	38.7	38.5	38.5	38.5	38.0	38.6	37.9	38.7	

(ii) Intra-Batch Variation.

This next experiment was designed to establish if different batches of Kaolin-platelet substitute reagent were of similar activity. Four freshly withdrawn plasmas were tested with different batches of Kaolin-platelet substitute reagent, using the kaolin-cephalin clotting time test. The results are tabulated in Table 3:2.

TABLE 3:2

THE INTRA-BATCH VARIATION
OF KAOLIN-PLATELET SUBSTITUTE REAGENT

Batch No.	KC17	KC18	KC19
	K.C.C.T. in Seconds		
Plasma A	40.6	44.5	40.3
B	35.3	40.6	34.7
C	41.2	46.4	42.4
D	32.4	35.8	32.2
Mean	37.4	41.8	37.4

There was a significant variation between some batches.

Accordingly, the same batch of material was used for a series of experiments.

(iii) Plasma Activation Rate.

The action of kaolin in the kaolin-platelet substitute reagent is to provide uniform activation of the contact factors (Factors XI and XII) in the initial stages of coagulation. The rate at which this activation occurs was therefore investigated.

Three plasmas were incubated with kaolin-platelet substitute reagent in a Factor VIII assay system for varying times up to 30 minutes. The results are summarised in Table 3:3. Maximal activation occurs within 10 minutes incubation and there was little change thereafter, up to 30 minutes. In the work described in this thesis, a 10 minute incubation period was used, although

in some assay experiments, the incubation period was increased to 15 or 20 minutes, and occasionally to 30 minutes.

These findings are similar to Hardisty et al (1962), Veltkamp (1968) and Hunter and Houghton (1968). The advantage of using conditions giving minimal clotting times is that small variations in kaolin concentration and incubation time have little influence on the final clotting times, thus facilitating more uniform results.

TABLE 3:3

EFFECT OF INCREASING INCUBATION TIME OF PLASMA
WITH THE KAOLIN-PLATELET SUBSTITUTE REAGENT IN THE
FACTOR VIII ASSAY SYSTEM.

Minutes of Incubation													
		1	2	4	6	8	10	12	16	20	24	28	30
Clotting Time in Seconds													
Plasma	A	92	87	77	73	-	70	72	72	71	70	-	74
	B	77	74	67	63	61	59	60	61	59	61	60	59
	C	76	71	62	57	58	56	55	55	56	54	-	56
Mean		82	77	69	64	(60)	62	62	63	62	62	(60)	63

Incubation Mixture: 0.1 ml. Factor VIII deficient plasma.

0.1 ml. normal plasma at a dilution of 1:10 in buffer.

0.2 ml. kaolin-platelet substitute reagent.

0.1 ml. 0.025 M calcium chloride.

Similar results were obtained using the kaolin-cephalin clotting time test.

(b) Technique of K.C.C.T.

0.1 ml. of test plasma sample kept on ice was added to three non-siliconised pre-warmed 55 x 11.5 mm. tubes, and incubated at 37°C for 1 minute.

0.2 ml. of kaolin-cephalin reagent was then added, the solution mixed thoroughly for 5 seconds and incubation continued for exactly 10 minutes, without further disturbance.

After this time, 0.1 ml. 0.025 M calcium chloride was added and the clotting time recorded in the Coagulometer. The mean of the three values observed was taken as the clotting time. In 96 determinations of the test in 90 normal individuals (age range 20-60 years), the mean value was 38.2 seconds ± 3.1 . The mean standard error for a single clotting time was ± 1.06 seconds. This corresponds to coefficient of variation of 3.02% for experimental error.

4. One Stage Prothrombin Time. (P.T.)

This test is a measure of the extrinsic system of coagulation as it is sensitive to changes on Factors II, V, VII and X. Plasma is clotted in the presence of an optimal concentration of brain extract and calcium chloride (Quick, 1935).

The method was as follows. To triplicate 3" x $\frac{5}{8}$ " clotting tubes was added 0.1 ml. of brain thromboplastin (phenolised rabbit brain thromboplastin - Diagnostic Reagents Ltd.) and warmed to 37°C. 0.1 ml. of citrated test plasma was then added, followed by 0.1 ml. of 0.025 M calcium chloride, and the clotting time recorded. The control plasma used for the test was a fresh frozen plasma pool that had not been stored for longer than 21 days (Hougie, 1963). Fresh normal plasmas were also frequently included as an additional check. The clotting times of these plasmas were invariably between 12 and 15 seconds.

5. Calcium-Thrombin Time. (T.T.)

Human thrombin, as supplied by the Blood Products Laboratory, Lister Institute, Elstree, Herfs., was used as suggested by Hardisty and Ingram (1965). The lyophilised thrombin was reconstituted as directed and stored in 0.1 or 0.5 ml. aliquots in small tubes at -40°C. For use, an aliquot was thawed and diluted 1:30 in 0.025M calcium chloride. The clotting time of normal pooled plasma using this dilution was between 12 and 15 seconds and was always checked on normal plasma with each separate test.

The test was performed by adding 0.1 ml. of 0.05 M tris buffer and 0.1 ml. of test or control plasma to a clotting tube and warming the solution to 37°C. 0.1 ml. of calcium-thrombin solution was then added and the clotting time recorded in triplicate tubes. The results were then compared with the control plasma.

6. Arvin Time. (A.T.)

This test makes use of a purified coagulant fraction ('Arvin') derived from the venom of the Malayan pit viper (*Agkistrodon rhodostoma*). This fraction has a thrombin-like activity, clotting fibrinogen directly, but unlike thrombin is not affected by the presence of heparin or heparin-like activity (Regoeczi, 1966; Esnouf and Tunnah, 1967).

Arvin was supplied (Twyford Laboratories Ltd.) in ampoules containing 0.9 ml. specific activity 82 units/ml. These units were arbitrary, but were of similar order to National Institute of Health (N.I.H.) thrombin units. The Arvin was buffered in 0.1 M sodium phosphate/saline (pH 7.0) and was stable when stored at 4°C.

For use in the Arvin time, the contents of 1 ampoule was diluted in 7.0 ml. of 0.05 M tris buffer. 1.0 ml. aliquots were then stored at -40°C in small tubes. When required an aliquot was thawed and 1.0 ml. of 0.025 M calcium chloride added and the solution kept at 4°C.

The test was performed in an identical manner to the calcium-thrombin time. To 0.1 ml. of 0.05 M tris buffer was added 0.1 ml. of test plasma and the solution warmed to 37°C. 0.1 ml. of calcium-Arvin was then added and the clotting time recorded in triplicate tubes. The results were then compared with a pooled normal plasma similarly treated.

7. Factor I (Fibrinogen) Assay.

Three methods were used.

- (a) Fibrinogen Assay as described by Ratnoff and Menzies (1951)
as modified by Alkjaersig (1960).

This was used as the standard reference assay with which other fibrinogen assay results could be compared. The technique was investigated and modified in this laboratory by Mackay (1967).

In this assay the test specimen is diluted in buffer containing E.A.C.A. (to prevent further proteolysis) and clotted with thrombin. The tyrosine released from the fibrin by hydrolysis with sodium hydroxide is estimated with Folin Ciocalteu's reagent, and the fibrinogen concentration calculated from a standard tyrosine curve.

The technique was as follows. Into each of four 5" x $\frac{5}{8}$ " non-siliconized test tubes was added 6.0 ml. of E.A.C.A.-saline, and 0.2 ml. of 2.5% calcium chloride solution. Into three of the four tubes was added 0.1 ml. of thrombin (100 units/ml. in 0.9% saline). To each tube was then added 0.2 ml. of test solution; the tubes mixed, covered with parafilm and left at 4°C for at least 12 hours to ensure complete coagulation.

On the following day a constant amount of well-washed glass Ballotini beads (diameter 0.15mm) was added to each tube, which was then centrifuged at 3,400 r.p.m. at 4°C for 5 minutes. The supernatant was then removed leaving a small amount of solution containing the glass beads and entrapped fibrin. Three washes were then performed using 0.9% saline, care being taken to ensure that beads were not lost on removal of the

supernatant. After the last wash, 0.4 ml. of 10% sodium hydroxide was added to the four tubes and mixed. Hydrolysis was completed by boiling the tubes in a water bath for 20 minutes with intermittent shaking. The tubes were cooled, and 0.6 ml. of trichloroacetic acid added. 2 ml. of 0.5 N sodium hydroxide was further added followed by 0.6 ml. of Folin and Ciocalteu's reagent (diluted one volume to two volumes of distilled water) and mixed well. Without delay, the tubes were centrifuged for 2 minutes at 4,000 r.p.m. at room temperature. Using the non-clotted tube of the quadruplicate as the blank, the tyrosine released was assessed by measuring the colour density developed in a Unicam S.P.600 at 650m μ using the red filter. The tyrosine released was then calculated from a standard tyrosine curve previously prepared and this reading converted to fibrinogen concentration by multiplying by 11.7. The mean difference between triplicate observations over a wide range of fibrinogen values, expressed as a percentage of the mean of these observations was 6.2%.

(b) Fibrinogen Assay as described by Ellis and Stransky (1961).

This assay was used as a routine assay, the method being rapid, reproducible and easy to perform.

In the assay, the increase in optical opacity resulting from the formation of fibrin, in thrombin clotted recalcified citrated plasma is measured, and compared with non-clotted plasma. The result can then be calculated by comparison with a fibrinogen standard curve previously prepared.

The technique was as follows. To 5.5 ml. of barbitone saline buffer (pH 7.2) in a glass test tube, 0.5 ml. of test plasma was added, the tube covered by parafilm and mixed by inversion. 3.0 ml. of this solution

was added to a Unicam cell ('test cell') and the remainder to another cell, hereafter referred to as the "blank" cell. Using a micro-titre pipette dropper (delivery volume 0.025 ml.), 2 drops of a calcium-thrombin solution was then added to the test cell, and mixed by inversion over parafilm for 15 seconds. The cell was then left undisturbed for 20 minutes at room temperature and the density evolved measured in a Unicam S.P.600, against the blank, at 470 ~~mμ~~ with the blue filter. The percentage plasma fibrinogen was then calculated from the standard curve. The standard error of this method, as calculated from a plasma assayed 48 times by two technicians over a period of 8 weeks, was $\pm 6.5\%$

(c) Fibrinogen Assay using the Thorpe Nephelometer.

The method is based on the use of the Thorpe Micro-Nephelometer (I.C.I. Pharmaceutical Division) illustrated in Fig.3:1, and investigated in this laboratory by Mackay (1967). Using the instrument, a beam of light is passed through a diluted plasma sample and the resultant light scattering by the particles measured by photometers incorporated in the machine. In the assay, the light scattering index (L.S.I.) of the test plasma is recorded, the plasma is then heated to 56° for 10 minutes, cooled, and the L.S.I. again measured. By reference to standard tables the difference in L.S.I. can be assessed and the percentage of heat precipitable fibrinogen present calculated.

Fibrinogen assays, using the Thorpe Nephelometer, the Ratnoff-Menzies assay and the Ellis-Stransky assay, on the same normal plasmas showed a good correlation and the results will be published elsewhere (Mackay, 1967).



Fig. 3:1. The Thorpe Micro-Nephelometer.

The mean standard error of this method of fibrinogen assay was 3.6% as assessed by 15 separate assays on one standard plasma.

8. Factor II (Prothrombin) Assay.

In this assay plasma prothrombin is progressively converted to thrombin in the presence of a diluted brain thromboplastin solution and calcium chloride. This clotting mixture is then sampled, at spaced intervals, into a fibrinogen solution and the clotting times are recorded. The total number of generated thrombin units can then be compared with a control normal plasma, similarly tested, and the percentage of prothrombin by comparison estimated.

The method was as follows. Brain thromboplastin (Diagnostic Reagents Ltd.) was diluted in 0.9% saline to give a one-stage prothrombin time of 25-30 seconds on a normal pooled plasma. The usual dilution was between 1:10 and 1:30.

Tubes containing 0.4 ml. of bovine fibrinogen (Diagnostic Reagents Ltd.) were placed in the water bath at 37°C. In another tube, a mixture was made of 0.4 ml. of test or normal plasma and 0.4 ml. of diluted brain. Recalcification was then performed with 0.4 ml. of 0.025 M calcium chloride and a timer started. The resulting fibrin clot was removed with an orange stick and at minute intervals 0.1 ml. of the incubation mixture was transferred to a tube containing fibrinogen. The clotting times were then recorded. Testing was continued until the clotting time exceeded 200 seconds.

To obtain quantitative results, the clotting time of the fibrinogen samples were converted to thrombin units from a thrombin/fibrinogen curve

prepared as follows :-

Tubes containing 0.4 ml. of bovine fibrinogen were placed in a water bath at 37°C. Dilutions of thrombin (Parke Davis & Co.) from 20 to 0.5 units/ml. were made and kept on ice. Clotting times of the fibrinogen were recorded by the addition of 0.1 ml. of thrombin dilution. The clotting times were then graphed against the units of thrombin and a thrombin/fibrinogen curve obtained. From this graph, the number of generated units of thrombin in an unknown test sample could be read off directly.

The percentage of prothrombin was then calculated, i.e.

$$\frac{\text{Thrombin units (test plasma)}}{\text{Thrombin units (control plasma)}} \times 100\%$$

The percentage prothrombin could also be calculated as recommended by Hardisty and Ingram (1965), i.e.

$$\% \text{ Result} = \frac{\text{Sum of Reciprocals of Clotting Time (test plasma)}}{\text{Sum of Reciprocals of Clotting Time (control plasma)}} \times 100\%.$$

9. Factor V (Proaccelerin) Assay.

The method was as described by Biggs and Macfarlane (1962). Artificially prepared substrate plasma was used; this being devoid of Factor V, but supplying Factors I, II, VII and X in excess. On the addition of thromboplastin, calcium and a plasma dilution, the rate of conversion of prothrombin to thrombin, reflected in the clotting time of the fibrinogen in the substrate plasma, is proportional to the amount of Factor V present.

Method:

(a) Preparation of substrate plasma.

400 ml. of blood was collected in 50 ml. of 0.1 M sodium

oxalate. The plasma was separated by centrifugation and incubated at 37°C until the one stage prothrombin time was longer than 90 seconds. The plasma was then stored in small aliquots at -40°C. This plasma substrate was exceptionally stable when prepared and stored in this way.

(b) Performance of Test.

Dilutions of pooled control frozen plasma were made in veronal buffer (pH 7.4), from 1/1000 - 1/10, and kept on ice. 0.1 ml. aliquots of substrate plasma were pipetted into a rack of coagulometer test tubes. Triplicate tubes were then warmed to 37°C and 0.1 ml. of dilution, 0.1 ml. of brain thromboplastin (Diagnostic Reagents Ltd.) and 0.1 ml. of 0.025 M. of calcium chloride added and the clotting time recorded in the Coagulometer. This was repeated for each dilution and a standard curve prepared, this being plotted on double logarithmic paper. Usually three dilutions of the test plasma were tested. The best parallel line was drawn against the standard curve and the percentage of Factor V read off the ordinate axis.

To test the reproducibility of this method ten analyses were performed on a single plasma sample. The mean standard error was found to be $\pm 6.5\%$

10. Factor VII (Proconvertin) Assay.

The method was based on the correction of the clotting time, using Factor VII deficient Beagle dog plasma, of dilutions of test plasma as compared with control plasma. The procedure is basically the same as the Factor V or Factor X assay, and is as described by Garner et al. (1967).

Dilutions of pooled and test plasma, ranging between 1/10 to 1/1000, were made in glyoxaline buffer (pH 7.3). 0.1 ml. of substrate Factor VII deficient plasma was added to triplicate clotting tubes and pre-warmed to 37°C. To this was next added 0.1 ml. of test or control plasma dilution and 0.2 ml. of thromboplastin/calcium chloride mixture (0.025 M.) and the clotting time recorded. The concentration of Factor VII in the test plasma could be ascertained by comparison with the standard curve prepared from the control plasma dilutions.

The human brain thromboplastin used in the assay was supplied by Dr. R. Garner (I.C.I.) as was the substrate plasma. Only limited experience was gained with the assay and no comparison could be made with results using a human substrate Factor VII deficient plasma. However, the assay gave consistent results and low values of Factor VII were obtained on plasma from patients undergoing anticoagulant therapy.

11. Factor VIII (Antihæmophilic Factor) Assay.

In the course of early experimental work, two methods for the assay of Factor VIII were investigated.

A. Two-stage Factor VIII Assay.

The first method is based on the thromboplastin generation test (Biggs, Eveling and Richards, 1955) and is the standard procedure used in the Clinical Coagulation Laboratory of the Edinburgh Royal Infirmary. This method, with its numerous modifications (Pitney, 1956; Biggs, 1957; Pool and Robinson, 1959), has given satisfactory results in the hands of these workers, and experience in the Coagulation Laboratory of this hospital has confirmed

these observations (Cumming et al., 1965).

Nevertheless, this assay is difficult to perform, requires considerable attention to both reagents and technique and exhibits marked day to day variation in results if performed by different technicians. It also requires the services of skilled technical staff if consistent results are to be obtained. Because of these features, the reagents were standardised as far as was possible and this improved the reproducibility of the assay.

(a) Modifications of Reagents.

(i) Use of a standard Bovine Preparation of Factor V.

A lyophilised Bovine Factor V reagent (Diagnostic Reagents Ltd.) was reconstituted with distilled water and then divided into aliquots of 0.1 ml. and stored at -40°C . Aliquots thus frozen, showed no significant loss of Factor V activity when stored for periods up to 2 months. For use, 2.9 ml. of 0.9% saline was added, and the solution kept on ice for up to 2 hours. This preparation of Factor V did contain detectable VIII activity on repeated assays of different batches, as assessed by a one-stage assay.

(ii) Use of a Standard Phospholipid Reagent.

The phospholipid finally chosen was brain phospholipid prepared according to Bell and Alton (1954). This was distributed in aliquots of 0.6 ml. and stored at -40°C . There appeared to be no loss of potency on storage for at least 6 months. For use, it was reconstituted with 2.4 ml. of 0.9% saline and also kept at 4°C .

(iii) Use of a Standard Dried Human Serum.

Two methods were used:-

(a) 200 ml. of human blood (without anticoagulant and in a non-siliconised M.R.C. bottle) was collected from a blood donor, incubated for 3 hours at 37°C, followed by 18 hours at 4°C. The serum was separated, spun for 1 hour at 2,000 r.p.m., and the top two-thirds of the serum collected. This was divided into 1.0 ml. aliquots into new test tubes and freeze dried. The tubes were then sealed and stored at -40°C. For use the material was reconstituted in 10 ml. of glyoxaline buffer, and left to "activate" for 24-48 hours at 4°C in the glass test tube. This serum was of consistent potency, and was the usual source of the serum factors in the two-stage Factor VIII assay.

(b) A serum was prepared as described by Kekwick and Walton (1964). This also gave satisfactory results, and was more convenient to use as it could be stored frozen in the activated form and thawed just prior to use.

(iv) Use of Aged A.C.D. Plasma as Substrate.

Out-dated plasma (over 21 days old) that had been collected in acid-citrate-dextrose anticoagulant was found to be an excellent substrate for use in the assay. Aliquots of 10.0 ml. were stored at -40°C and thawed as required. Low levels of Factor VIII were present in this substrate.

(b) Technique of Assay.

The technique of assay was that described by Biggs et al. (1955).

The method is based on the thromboplastin generation test and consists of testing three dilutions of both adsorbed control and test plasma. Minimum clotting times obtained in this system are proportional to the amount of Factor VIII present, as all other factors are present in excess. Sub-sampling of the incubation mixture is performed twice when a stable plateau of thromboplastin formation has been reached.

The mean 95% confidence limits of 35 randomly selected routine Factor VIII assays, of values lying between 20% and 300% of normal plasma, was 75% to 133% of the observed result. The widest 95% confidence limits for an assay was 55% to 183%, the narrowest, 90% to 111%.

B. One-stage Factor VIII Assay.

The second type of Factor VIII assay available is the method based on the correction of clotting time that occurs when a dilution of a test sample is added to a plasma congenitally deficient in Factor VIII. These assays are referred to as "one-stage" assays, as sub-sampling of an incubation mixture is not required.

This method of assay has been improved considerably by the standardisation of contact activation in the incubating mixtures, and by the use of a phospholipid which eliminates variations in platelet numbers between tubes. The method selected was similar to that used by Hardisty and Macpherson (1962) as modified by Veltkamp (1966) but with the following changes in techniques.

- a) Kaolin-cephalin reagent was used as a combined source of contact activator and phospholipid.

- (b) All dilutions were made in tris buffer pH 7.4.
- (c) Four dilutions of both test and control plasma were tested in duplicate and in balanced order to reduce the effect of any "drift" in the reagents used (Ingram, 1966).
- (d) All dilutions were kept on ice until used, and substrate deficient plasmas were from haemophilics of proved very low levels of Factor VIII.
- (e) Small aliquots of substrate plasma were stored for up to 3 weeks, before being discarded. In some experiments, out-dated Factor VIII deficient plasma was reinforced with Bovine Factor V (Diagnostic Reagents Ltd.) and the results of these assays were in good agreement with assays using fresh substrate plasma.

Assay Techniques.

A series of disposable 55 x 11.5 mm. tubes kept on ice were filled successively with 0.1 ml. freshly thawed substrate Factor VIII deficient plasma and 0.1 ml. of dilution of test or control plasma. After an incubation period of 1 minute at 37°C, 0.2 ml. of kaolin-cephalin reagent was added, and incubation continued for a further 10 minutes. 0.1 ml. of 0.025 M calcium chloride was then added, and the clotting time measured in an automatic Coagulometer, in duplicate, or in some experiments in triplicate. Dilutions of test plasma were so arranged that clotting times of both test and control plasmas were similar, to avoid extrapolation of the final standard reference curve. The coagulation times obtained with the various concentrations of both the reference and test plasmas were plotted on semi-logarithmic paper, the lines of best fit drawn, and the percentage values

of the test sample read off directly. In many experiments, however, confidence limits and percentage values were calculated statistically by computer.

The mean 95% confidence limits for 76 randomly selected Factor VIII assays of values lying between 0 to 600% of normal standard plasma was 81-125% of the observed result. The widest confidence limits were 61% to 165%, the narrowest 95% to 106%. These confidence limits are similar to that of Simone et al. (1967), and Veltkamp (1968), both of whom used automatic end point recorders. These confidence limits include the experimental errors of both the standard curves and test plasmas, the slopes of the dose response curves with regard to the degree of parallelism and the related potency of the test versus control plasmas. The experimental error, as assessed by 12 separate determinations on a single plasma (mean value 103%) was 6.3%. This compares favourably with Veltkamp (1966) who found his experimental error to be 5.7%.

This one-stage assay was, in our hands, technically easier and more reproducible than the two-stage assay (see confidence limits) and was thus used extensively. Occasional anomalies occurred, but on repetition of the assay, this was nearly always due to either technical fault or unsatisfactory reagents. It was found that with consistent practice, the 95% confidence limits of the assay improved, i.e. in the most recent work, the mean of the confidence limits was 83% to 117% of the observed results.

The two-stage and one-stage assays were performed on 25 separate frozen plasma samples. There was good agreement between the two methods,

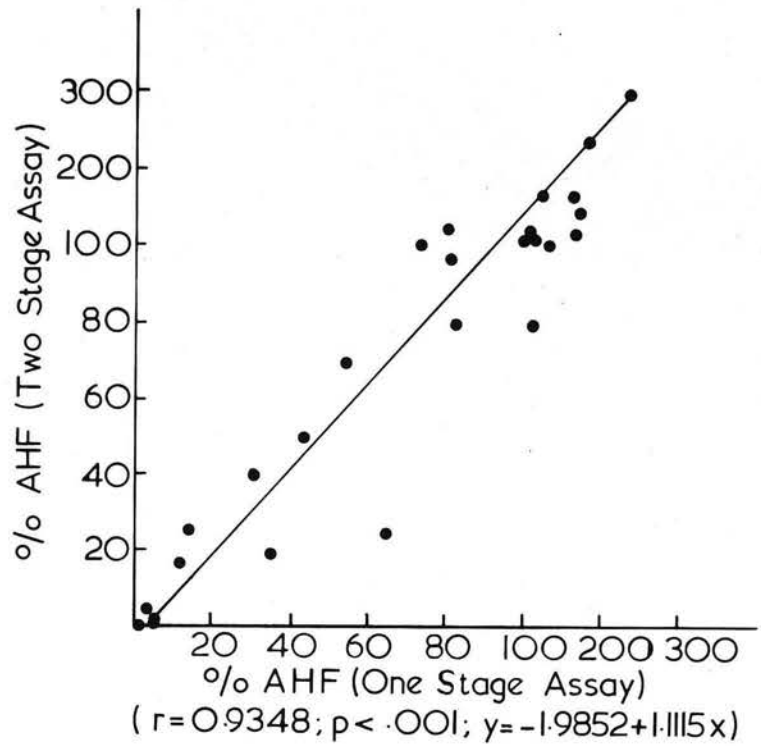


Fig. 3:2. Correlation between "One-stage" and "Two-stage" Factor VIII Assay Systems, on 25 Separate Plasmas.

with the two-stage assay tending to give slightly higher values of Factor VIII than the one-stage method (Fig. 3:2) ($r = 0.9348$, $p < 0.001$).

12. Factor IX (Christmas Factor) Assay.

The technique used was based on the one-stage assay method described by Hardisty and Macpherson (1962). Substrate plasma deficient in Factor IX was used, and to this was added dilutions of test or normal plasma, the solution activated by the use of a kaolin-platelet substitute reagent, and the clotting time recorded.

The method was identical in procedure to the one-stage Factor VIII assay apart from the preparation of the substrate plasma.

For substrate plasma, only plasma from patients with severe Factor IX deficiency was used. This was stored in small aliquots at -40°C . For use, it was mixed with an equal volume of adsorbed citrated fresh normal plasma, the latter supplying supplementary Factor V and Factor VIII, which may have deteriorated on storage of the substrate plasma.

The mean 95% confidence limits for this assay for 25 randomly selected assays with values lying between 0-136% normal plasma was 79-122%. The reproducibility of the technique was checked by assaying the same plasma sample eight times. The mean value was $100 \pm 9.1\%$.

13. Factor X (Stuart Prower) Assay.

The method was as described by Denson (1961). Commercially available Factor X deficient substrate plasma, prepared from filtered ox plasma, is also deficient in Factor VII, but contains Factors I, II and V.

Russel's Viper Venom (R.V.V.) can convert fibrinogen to fibrin in the presence of these factors and phospholipid, in the absence of Factor VII. The clotting times so obtained are, under specified conditions, proportional to the amount of Factor X present.

The technique was as follows. Accurate dilutions from 1:10 to 1:1,000 of pooled normal plasma were made in veronal/saline buffer (pH 7.4). To triplicate coagulometer clotting tubes was added 0.1 ml. of Factor X deficient substrate plasma, and 0.1 ml. of plasma dilution. The tubes were then warmed to 37°C and 0.1 ml. of Russel's Viper Venom/Cephalin Mixture added. At exactly 30 seconds after this addition, 0.1 ml. of 0.025 M calcium chloride was used, and the clotting time recorded in the Coagulometer. The test plasma dilutions were usually tested at two dilutions (1:10 or 1:20). The percentage Factor X present could then be calculated by comparison with the standard curve when charted on double logarithmic graph paper.

The reproducibility of this assay method was tested by assaying the Factor X level ten times on a single normal plasma. The mean value was $100 \pm 5.3\%$.

14. Factor XII (Hageman Factor) Assay.

The method was identical to that of the Factor VIII and Factor IX assays, except that siliconized tubes were used both for preparing dilutions and also for the clotting procedures. Factor XII deficient substrate plasma was kindly provided by Dr. Loeliger, Leiden, Holland. Dilutions in veronal/saline were usually started at not less than 1:40 as advised by Veltkamp (1967), and dilutions of the test plasma were so chosen as to avoid extra-

polation of the standard control curve.

The mean 95% confidence limits for this assay, calculated from 20 assays was 77% to 131% of the observed results. Six assays performed on a normal pooled plasma gave a mean value and standard deviation of $100 \pm 12\%$.

15. Factor XIII (Fibrin Stabilizing Factor) Assay.

The method was that as described by Das (1968). Fibrin clots, obtained from a test plasma and standard substrate fibrinogen in the presence of mono-iodoacetic acid, were treated with the solvent mono-chloroacetic acid. The residual fibrin clots were hydrolysed and the tyrosine release measured in a spectrophotometer. The percentage of Factor XIII present could then be calculated by comparison with the normal standard from a previously prepared tyrosine calibrated curve.

The technique is similar to that of the fibrinogen assay as described by Ratnoff and Menzies (1961), apart from the initial stages of preparation of the test specimen.

16. Assay of Contact Activity (Factors XI and XII).

In this assay, test plasma is exposed to celite, the adsorbed contact product subsequently eluted, dilutions made in saline, and these added to fresh normal non-glass contacted plasma and the clotting times recorded. The percentage activity can then be calculated from comparison with a normal plasma, similarly treated.

The modified method used was similar to that described by Nossel (1964). To two volumes of normal platelet poor non-contact plasma,

collected in plastic tubes, was added one volume of celite 512 (100 mgm/ml. in 0.85% saline). This was incubated at 37°C for 10 minutes with constant agitation and the celite deposited by centrifugation at 2,500 r.p.m. for 5 minutes. The supernatant plasma was carefully decanted and the celite resuspended in normal saline of volume approximately equal to that of the original plasma. The celite was again deposited by centrifugation and the supernatant decanted off and discarded. The procedure was repeated three times. The washed celite was then suspended in a volume of 10% saline equal to the volume of the original plasma. The mixture was kept at 37°C for 10 minutes with constant mixing and the celite removed by centrifugation. The supernatant was dialysed (Visking casing) at 4°C with continuous stirring (magnetic stirrer) against exactly ten times the eluate volume of distilled water. The dialysed eluate was stored in aliquots at -40°C until assayed.

In the assay, dilutions of the eluate were made in saline in silicone treated tubes. Mixtures (in silicone treated tubes) were then made as follows :-

0.1 ml. fresh normal non-contact plasma

0.1 ml. Bell and Alton Platelet substitute.

Incubated for exactly 1 minute.

0.1 ml. eluate dilution

0.1 ml. 0.05 M calcium chloride was then added, and the clotting time recorded.

The resultant clotting times were plotted on double logarithmic graph paper and the percentage contact factor activity calculated from the control contact product eluate curve.

17. Heparin Precipitable Fraction (H.P.F.) Assay.

A modified assay system was devised, based on the method described by Smith (1957). Heparinised plasma was incubated at 4°C overnight to allow precipitation of the H.P.F.; the precipitate was then centrifuged and washed with a buffer to remove extraneous protein, and then hydrolysed with a strong alkali. The tyrosine released from the H.P.F. was then estimated with Folin-Ciocalteu's Reagent in a spectrophotometer.

A. Technique of Assay.

5.0 ml. of blood was collected in polystyrene tubes containing 0.1 ml. heparin (1,000 u/ml. saline) and mixed well. This was then centrifuged at 4,000 r.p.m. (R.C.F. 2,652) for exactly 15 minutes at room temperature. The plasma was separated and aliquots of 0.5 ml. were added to three non-siliconized acid cleaned 5" x $\frac{5}{8}$ " test tubes. The tubes were covered with parafilm and placed at 4°C overnight (18 - 24 hours).

On the following day the plasma was centrifuged at 3,200 r.p.m. (R.C.F.) for 15 minutes at 4°C, and the supernatant plasma decanted off. The precipitate was then washed three times using approximately 5.0 ml. per tube of 0.05 M phosphate buffer (pH 7.4) at 4°C, for each wash. After the third wash the tubes were drained thoroughly. Hydrolysis was carried out by adding 0.4 ml. 10% sodium hydroxide and boiling in a water bath for 20 minutes. The tubes were then cooled under tap water and 0.6 ml. of 5% trichloroacetic acid, followed by 2 ml. of 0.5 N sodium hydroxide, added. 0.6 ml. of Folin-Ciocalteu's Reagent (diluted 1:2 in distilled water) was added and the solution centrifuged for exactly 1 minute at 2,500 r.p.m.

(R.C.F. 1,036) at room temperature, to remove any precipitate which occasionally appeared. The colour intensity developed was read in a Unicam S.P.600 at 650 m μ , using the red filter against a reagent blank. The H.P.F. concentration was calculated from a standard tyrosine curve previously prepared.

B. Investigations of Assay Techniques.

(a) Concentration of Heparin.

The effect of heparin concentration on the yield of H.P.F. was investigated. Blood collection tubes containing between 2 and 80 units of heparin per ml. of whole blood were prepared and the H.P.F. yield measured on the same plasma.

The results of four separate experiments are illustrated in Fig. 3:3 and Table 3:4. As heparin concentration increased, the yield of H.P.F. also rose. The slope of increase also varies between plasmas (Fig. 3:3), two of the four plasmas assayed demonstrating a marked change with increasing heparin concentration while changes in the other two plasmas were less pronounced. A concentration of 20 units of heparin per ml. of whole blood was selected for routine use.

(b) Incubation Time of Heparinised Plasma.

In two separate experiments, plasma was stored at 4°C for periods varying between 16 and 48 hours. There was no change in the amount of H.P.F. obtained with increasing storage time, the yield at 16 hours being similar to that at 48 hours. Therefore, in the routine assay, the plasma

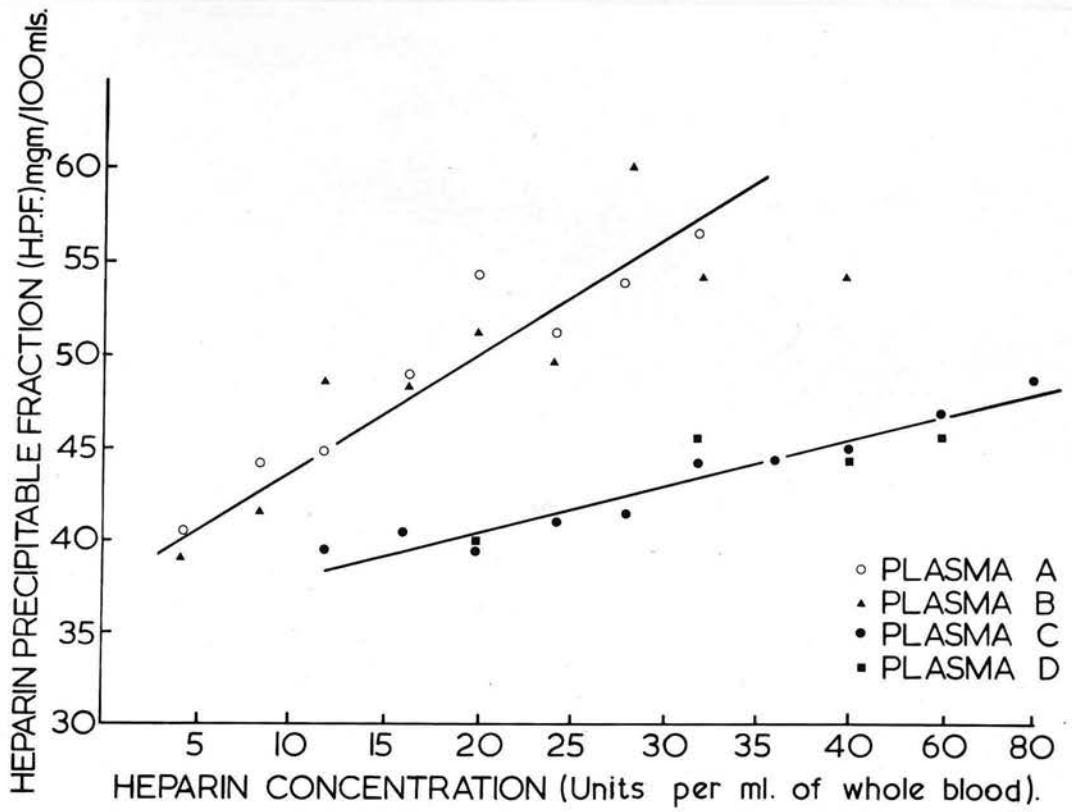


Fig. 3:3. Effect of varying concentrations of heparin on the yield of H.P.F.

TABLE 3:4.

THE EFFECT OF VARYING CONCENTRATIONS OF HEPARIN
ON THE YIELD OF H.P.F.

Units Heparin/ ml. blood	H.P.F. mgm/100 ml.			
	Plasma 1	Plasma 2	Plasma 3	Plasma 4
4	-	-	39.2	40.5
8	-	-	42.5	44.1
12	-	39.4	47.3	44.8
16	-	40.4	47.6	47.1
20	39.6	39.8	51.4	54.6
24	-	41.0	49.8	51.5
28	-	41.2	60.8	54.2
32	45.4	44.2	54.6	56.8
36	-	44.4	-	-
40	44.3	44.9	54.6	-
60	45.6	46.7	-	-
80	-	47.4	-	-

was left overnight and the storage time by this method varied between 18 and 24 hours.

(c) Storage of Heparinised Plasma at -40°C .

Heparinised blood was centrifuged, the plasma separated, one sample assayed for H.P.F., and the remainder frozen at -40°C . This latter plasma was thawed completely at 37°C , and then assayed for H.P.F.

In Table 3:5 is shown the results of ten experiments :-

TABLE 3:5
RESULTS OF TEN PLASMAS ASSAYED FOR H.P.F. BEFORE AND AFTER
STORAGE AT -40°C

Plasma	Fresh Plasma	Frozen Plasma
	H.P.F. - mgm/100 ml.	
1	109.8	167.7
2	57.1	66.9
3	55.4	56.5
4	37.7	44.7
5	193.0	122.9
6	52.6	56.3
7	49.5	50.9
8	58.5	59.7
9	90.1	83.9
10	50.2	45.1
Mean	75.4	75.5

(t = 0.0072, p = N.S.)

There was no statistically significant difference between the paired results ($t = 0.007$). Heparinized plasma can thus be frozen and assayed at a convenient date; the longest period plasma was stored at -40°C was three weeks.

(d) Technique for Precipitate Washing.

H.P.F. was washed either two or three times with buffer. Two washes were found to be sufficient, although three washes were used routinely.

(e) Type of Tube used in the Assay.

H.P.F. was assayed in both siliconized and non-siliconized tubes. There was no significant difference in H.P.F. values between the type of tube used.

(f) Cryofibrinogen in Oxalated and Citrated Plasma.

4.5 ml. of blood was collected in either 0.5 ml. of 3.8% w/v sodium citrate or 0.5 ml. of 1.34% w/v potassium oxalate. The plasma was separated and assayed as for H.P.F.

In four separate experiments, the mean value of the cryofibrinogen obtained in the citrated plasma was 3.9 ± 0.8 mgm.%, and in the oxalated plasma 5.5 ± 2.7 mgm.%.

(g) Errors of the Method.

(i) Errors between operators.

Six samples were assayed separately by two technicians (Table 3:6). There was no significant difference between the paired results.

Usually, however, to ensure reproducibility of results within a series of experiments, the same technician performed the assays.

TABLE 3:6.

RESULTS OF SIX SEPARATE PLASMAS ASSAYED FOR H.P.F.
INDEPENDENTLY BY TWO TECHNICIANS

Plasma	Technician 1	Technician 2
	H.P.F. - mgm/100 ml.	
1	43.2	35.6
2	47.4	42.5
3	50.3	44.4
4	31.3	35.7
5	31.4	34.5
6	30.6	37.9
Mean	39.0	38.4

(ii) Assessment of Percentage Error of the Method.

Each sample was assayed in triplicate. The mean of the two closest observations was taken as representing the level of H.P.F.. The mean difference between the triplicate results, over a wide range of H.P.F. values (12.9 - 156 mgm/100 ml.), expressed as a percentage of the mean of these results was 5.6%. This error is the mean value of the routine laboratory assay, and includes errors of both day to day alterations in assay technique and individual technician variation.

C. Normal Values of H.P.F.

In 59 observations on 50 normal subjects, age range 20-58 years, the mean level of heparin precipitable fraction was 44.5 ± 10.9 mgm./100 ml.

CHAPTER III

FIBRINOLYTIC ASSAY TECHNIQUES

1. Plasminogen Assay.

The method was as described by Remmert and Cohen (1949), as modified by Alkjaersig (1959). Antiplasmins are removed by incubating the plasma with acid, the acid is then neutralized with alkali, and streptokinase added to convert the plasminogen to plasmin. The plasmin evolved is assayed with casein, the amount of tyrosine released being proportional to the concentration of plasmin, and consequently plasminogen, present.

A. Assay method.

To 0.5 ml. of plasma, in duplicate or triplicate and using disposable plastic tubes, was added 0.5 ml. $N/6$ Hydrochloric acid, and the mixture left at room temperature for 15 minutes. 0.5 ml. of $N/6$ sodium hydroxide was then added followed by 1 ml. of 0.1 M phosphate buffer, 0.5 ml. of Streptokinase (2,000 u/ml. in 0.1 M phosphate buffer), and 2.0 ml. of casein solution. These reagents were well mixed and 2 ml. withdrawn and added to 2ml. of 10% trichloroacetic acid. The remaining 3.0 ml. were incubated at $37^{\circ}C$ for 60 minutes, and at the end of this time 3.0 ml. of 10% trichloroacetic acid was added. The precipitates were removed by centrifugation for 2 minutes at 3,400 r.p.m. and the supernatant filtered through Whatman's No. 1 Filter Paper. For the hydrolysis stage 1.0 ml. aliquots was removed from each tube and added to a tube containing 5 ml. of 0.5 N sodium hydroxide and 1.5 ml. of 5% trichloroacetic acid. An

addition of 1.5 ml. of Folin and Ciocalteu's reagent (diluted 1 part of Folin's to 2 parts of distilled water) was then made and after 15 minutes at room temperature the optical density measured, reading the zero sample against the 60 minute sample in the Unicam S.P.600 at 650 m μ and using the red filter. The tyrosine released in mgm. was calculated for a standard curve and one casein unit was equivalent to 180 μ g of tyrosine released in one hour.

The mean-percentage error of this method in 50 assays covering a wide range of normal values was 2.9%. The normal mean value, as assessed on 36 random donors was 3.67 ± 0.6 c.u./ml. with a range lying between 2.74 and 5.54 c.u./ml.

B. Preparation of Plasminogen standard curve.

A solution of tyrosine (British Drug Houses Ltd.) containing 72 mgm. per 100 ml. of 0.1 N hydrochloric acid was made up. Doubling dilutions of this solution were made in 0.1 N hydrochloric acid to give solutions equivalent to 2.0, 1.0 and 0.5 casein units per ml. activity. The initial solution was taken as being equivalent to 4 casein units per ml.

To 0.5 ml. of each dilution was added 0.5 $N/6$ hydrochloric acid, 0.5 $N/6$ hydrochloric acid, 0.5 $N/6$ sodium hydroxide, 1.5 ml. of 0.1 M phosphate buffer (pH 7.6), 2.0 ml. of 5% casein and 5 ml. of 10% trichloroacetic acid. 1.5 ml. of Folin and Ciocalteu's Reagent was then added (diluted 1 part to 2 parts distilled water) and after 15 minutes at room temperature the optical densities were read against a similarly prepared blank tube. The graph correlating optical density against activity in casein units was then plotted.

2. Euglobulin Lysis Time Assay.

This assay is regarded as being a measure of circulating plasminogen activator, in the virtual absence of fibrinolytic inhibitors. Euglobulin, prepared from plasma by acidification and dilution, contains plasminogen activator, plasminogen and fibrinogen as well as other plasma proteins. Fibrinolytic inhibitors are discarded in the supernatant. The euglobulin precipitate is redissolved in buffer, clotted with thrombin; and the time taken for lysis to occur observed and recorded. The method is as described by Cash (1967).

Technique of Assay.

Venous blood, collected by the usual technique and kept on melting ice for not longer than 10 minutes, was centrifuged at 3,400 r.p.m. for 20 minutes at 4°C. The upper third of the plasma was removed and 1.0 ml. added to a siliconized centrifuge tube containing 19.0 ml. of a dilute solution of acetic acid. The pH was adjusted to 6.0 with 0.25% acetic acid and euglobulin precipitation continued for 10 minutes at 4°C. The tube was then spun at 3,400 r.p.m. for 20 minutes at 4°C, the supernatant decanted, the inner side of the tube wiped dry with a tissue and the precipitate resuspended in 1.0 ml. of barbiturate buffer (pH 7.4). 0.24 ml. of this solution was transferred to three 3" x $\frac{5}{8}$ " siliconized tubes in triplicate and 0.24 ml. of thrombin solution 5 u/ml. added immediately. Lysis was recorded in an automatic clot lysis recorder (Cash and Leask, 1967). The mean percentage error for this method was 2.4% \pm 0.5.

Euglobulin lysis times were, in some studies, converted to units using the formula $\frac{1,300}{T}$ of Januszko and Dubinska (1964), where T was the

euglobulin lysis time in minutes.

In physiological experiments, where the presence of plasmin was unlikely, the units were referred to as "plasminogen activator units". In other situations, where the presence of plasmin could not be excluded, the term "euglobulin lysis activity units" was used.

3. Fibrin Plate Assay Technique.

Fibrin plates were prepared by a modification of the method of Astrup and Mullertz (1952). Both human and bovine fibrin plates were used, but the human fibrin preparations proved to be more sensitive (in terms of area lysed). These unheated plates were regarded as a semi-quantitative measure of plasminogen activator although plasmin, if present in the test assay sample, would also cause fibrin lysis. This assay technique was of good confirmatory evidence for the euglobulin lysis time assay, although not as sensitive.

Technique of Assay.

10 ml. of 0.15 gm.% of human fibrinogen (Kabi) in tris buffer was pipetted into a disposable plastic petri dish and clotted, using a circular motion, with 0.2 ml. of a thrombin solution 50 u/ml. also in tris buffer, on a level table. After 20 minutes, when clotting was complete, 0.03 ml. samples of the test material, plasma or euglobulin, were dropped in triplicate on to the fibrin films. Plates not in use were stored at +4°C. The plates were then incubated from between 20-24 hours at 37°C, and at the end of this time, the perpendicular diameters of the lysed areas measured. The sensitivity of the plates were checked daily by using a standard solution

of urokinase (3.0 u/ml. in tris buffer) on a separate fibrin plate. The mean percentage error of this method was $12.4\% \pm 0.61$.

4. Fibrinogen/Fibrin Degradation Products (F.D.P.) Assay (I).

A. Introduction.

The lysis of fibrinogen or fibrin by plasmin either in vivo or in vitro results in the formation of degradation products, some of which are incoagulable with thrombin, resistant to further plasmin digestion, but share a common antigenicity with fibrinogen. Methods to identify these breakdown products have attracted considerable interest as they present incontrovertible evidence of active fibrinolysis by proteolytic enzymes.

It is possible to detect F.D.Ps. by simple tests such as the thrombin time but this method is of poor specificity and sensitivity. Immunological methods of identification such as immunodiffusion and immunoelectrophoresis have also been used, but again these tests are at best only semi-quantitative. (Ferreira et al., 1963; Nيلهn and Nilsson, 1964). It was not until the utilization of a tanned red cell haemagglutination immunoassay technique such as used in the bioassay of chorionic gonadotrophin (Wide, 1962), growth hormone (Grumbach and Kaplan, 1962), and fibrinogen (Fox et al., 1964), that accurate assessment of F.D.P. concentrations became available.

The method developed in this laboratory is similar to that described by Merskey et al. (1966) and investigated fully by Das (1968). Sensitised tanned sheep red cells are conjugated with fibrinogen and these can be agglutinated by fibrinogen antisera. This haemagglutination reaction is inhibited by the presence of lytic products of fibrinogen or fibrin or by

fibrinogen itself. This assay is both specific and sensitive and can accurately quantitate small amounts of antigenic material. It has the disadvantage of not being specific for fibrin degradation products, but reacts also to fibrinogen and other clottable fibrinogen derivatives. Thus considerable care is needed in the preparation of samples for assay to ensure removal of all traces of other antigenically active material. This defect cannot be overcome by using a specific F.D.P. antisera as this is antigenically cross reacting with fibrinogen.

B. Technique of Tanned Red Cell Haemagglutination Inhibition

Immunoassay (T.R.C.H.I.I.).

(a) Preparation of fibrinogen coated, sensitised sheep red cells.

25 ml. of fresh sheep red cells (Burroughs Wellcome & Co.) stored in Alsever's Solution, were washed four times with an equal volume of 0.9% saline. The packed cell volume (P.C.V.) was then measured by centrifuging the cells at 1,000 g for exactly five minutes in a graduated Pyrex centrifuge tube. An 8% suspension of the cells was made in 0.9% saline and an equal volume of 3% formal saline added. The cells were next incubated at 37°C for 20-22 hours, with gentle agitation using a magnetic stirrer.

After this time, the formalised cells were washed four times with fresh distilled water, followed by three washes with 0.15 M phosphate buffer (pH 6.4). The P.C.V. was again measured as outlined above. A 2% cell suspension was made in 0.15 M phosphate buffer (pH 6.4) and equal volume of working solution of tannic acid (1:40,000) added. The mixture

was heated in a water bath to 56°C and incubated for 1 hour with gentle mixing at 10 minute intervals. The tanned cells were then washed three times with 0.15 M phosphate buffer (pH 6.4) and the P.C.V. measured. Using 0.1 M citrate buffer (pH 6.4) a 4% cell suspension was made and an equal volume of a $2\mu\text{gm/ml}$. solution of fibrinogen added. The mixture was then further incubated at 37°C for 60 minutes with gentle stirring at 15 minute intervals.

A further three washes of the cells with 0.15 M citrate buffer (pH 6.4) was then made and the P.C.V. estimated. From these cells a 2.5% cell suspension was made in diluting fluid and these aliquots were stored in plastic disposable tubes at 4°C . After 3-4 days' storage the diluting fluid was replaced.

For daily use, the 2.5% suspension of cells was transferred to small plastic tubes, each aliquot of 2.5 ml. providing sufficient suspension for use on one microtiter plate. Before use, these cells were washed three times; twice with citrate buffer (pH 6.4) and once with diluting fluid to ensure that any fibrinogen eluted off the cells on storage was not present in the assay system.

(b). Collection of Test Samples.

(i) Serum.

5 ml. of whole blood were collected in clean glass tubes containing 0.1 ml. Trasylol (5,000 K.I. units/ml.), and mixed well. These tubes were incubated at 37°C for up to 12 hours, but as a routine 4 hours. The serum was separated by centrifugation and stored at -40°C in small aliquots.

(ii) Plasma.

4.5 ml. of whole blood was collected into plastic tubes containing 0.5 ml. of 3.8% sodium citrate and 0.1 ml. Trasylol (5,000 K.I. units/ml.), the plasma separated by centrifugation, and stored in aliquots at -40°C .

(c) Preparation of Test Samples.

This stage was crucial in the assay, as incomplete removal of the fibrinogen in the test sample would invalidate the results of the assay. The serum samples were thawed, one-eleventh volume of thrombin (100 u/ml. in saline) added and then incubated at 37°C for 1 hour. When plasma samples were assayed, one-eleventh volume of thrombin was added and then incubated at 37°C for 2-4 hours. Resultant clots were removed and a further eleventh volume of thrombin added and incubated for another hour. Sera were then separated by centrifugation, prior to the adsorption stage.

(d) Adsorption of Test Samples.

This step was necessary to remove any naturally occurring agglutinating anti-sheep cell substance present in the test sera.

Sheep red cells (Burroughs Wellcome & Co.) in Alsevers solution and stored at 4°C were washed four times with 0.9% saline. Cells showing excessive haemolysis after the first wash were discarded. After the fourth wash, the cells were packed by prolonged centrifugation and as much as possible of the supernatant saline removed.

To each 2 volumes of test sample, 1 volume of packed sheep cells were added. The suspension was well mixed and incubated overnight

at +4°C. The following day the suspension was centrifuged, the adsorbed serum separated and either stored in small aliquots at -40°C or assayed immediately.

(e) Assay of Test Samples.

(i) Dilution of the test samples.

Microtiter plates (Micro titer*, Cooke Engineering Co.) carefully washed as outlined in the appendix, were labelled, and using a microtiter dropper (delivery volume 0.025 ml.) into the first plate cup was added either 0.05 ml. of test sera, or 0.025 ml. of sera and 0.025 ml. of diluting fluid. Precalculated volumes of diluting fluid were then added to the remaining cups, depending upon the dilution steps required. Usually doubling, with or without intermediate dilution steps were made. Using the microtiter multiple diluting gun (delivery volume of each head 0.025 ml.) and starting at the first cup, the dilutions were prepared. Positive and negative controls were included with each plate.

Fibrinogen standards (1.25 mgm/ml., 2.5 mgm/ml., 5.0 mgm/ml.) were included with each assay and these were always diluted in an identical method as above. The fibrinogen content of these standards was checked frequently using the method of Ratnoff and Menzie (1951), as modified by Alkjaersig (1960). Small aliquots of the non-diluted standards were stored at -40°C, thawed, and diluted as required.

(ii) Addition of Fibrinogen antisera.

To each cup of the microtiter plate, and using the microtiter dropper, 0.025 ml. of the selected anti-human, rabbit fibrinogen antisera (Hoechst Pharmaceuticals Ltd.) was added. The plates were mixed; and the

test sample/antisera mixture incubated at 4°C for either 4 or 24 hours.

(iii) Addition of sensitised fibrinogen coated sheep red cells.

After incubation as outlined, one drop (0.025 ml.) of a previously thrice-washed 2.5% sensitised, fibrinogen coated sheep red cell suspension was added to each microtiter cup. The plates were then mixed and stored at $+4^{\circ}\text{C}$, without disturbance, overnight.

(iv) Reading of F.D.P. Assay Results.

The plates were removed from the refrigerator and left at room temperature for 30 minutes before reading. As far as was possible, an identical lighting source was used daily. The end point of both the fibrinogen standards and the test sera was taken as the last sharp button of non-agglutinated cells appearing in each row of the microtiter plate. Results were scored as -, +, $\bar{+}$, or \pm . Each plate was read twice in reverse order.

(v) Calculation and Control of Results.

The mean sensitivity of the system for that day was then calculated from the fibrinogen standards by comparing the last cup showing inhibition in the unknown series, with the last cup showing inhibition in the standard. From this, and the known dilution, the F.D.P. values of the serum samples could be obtained. A control serum was included with each separate assay as a further check against gross errors in technique. Sera samples from normal subjects were frequently included in the test samples as an additional check on the method.

C. Standardisation of the T.R.C.H.I.I. Assay

Initial experience with this assay suggested that the sensitivity of the system was varying considerably from day to day and that reproducibility of

results on some test samples, was not always satisfactory, when the test was utilized as a routine procedure. The following facets of test were investigated and are complementary to similar studies carried out by Das (1968).

(a) Preparation of test samples.

(i) In initial work, reliance was placed on the spontaneous clotting of whole blood for 4 hours at 37°C but, in some sera from patients with pathological conditions, e.g. haemophilia, the conditions for clotting required extension as fibrinogen conversion was not complete in this time. Accordingly all sera samples were reclothed with thrombin as a routine procedure. Similar considerations apply to trasylol citrate samples and these were usually clotted twice with thrombin.

This procedure was adhered to rigidly and improved the reproducibility of the assay on pathological samples. It was also found that specimens containing concentrations of heparin above 1.0 u/ml. of whole blood, required an extended period of coagulation and were thus usually clotted for 4 hours at 37°C , and then overnight at 20°C with additional thrombin added.

(ii) Dilution of the test sera by varying volumes of added sheep red cells in the adsorption stage could affect the absolute result, especially in the physiological range. The volume of sheep red cells added was thus standardised at 1 volume of cells to 2 volumes of sera, as measured by pipette. When adsorption was incomplete, the sample was adsorbed twice.

(iii) All initial studies were performed on fresh adsorbed

serum. A study was made to ascertain if adsorbed sera could be frozen and re-thawed when required for assay. Ten separate sera were prepared by three different methods as tested in Table 3:7. It can be seen that freezing and subsequent thawing did not cause a change in the F.D.P. level.

Therefore as a routine procedure, batches of frozen test sera were thawed, reclotted, adsorbed, and refrozen until required for assay. This method facilitated handling of large numbers of specimens.

(b) Preparation of Sensitised Cells.

(i) Washing of sensitised cells prior to use in assay.

In some vials of stored cells small amounts of antigenically active material, probably eluted fibrinogen, was detected in the supernatant. Cells were therefore routinely washed three times before use.

(ii) Preparation of human sensitised cells.

Use of human cells would obviate the adsorption stage and could possibly be more sensitive than sheep cells (Weir, 1967).

Cells from a Group O Rhesus Negative donor were obtained, sensitised and coated by the usual method (one run only was performed).

These cells were tested in the assay system and were found to be of similar sensitivity to the routinely prepared sensitised sheep cells. However, end point formation was not as satisfactory and it was considered that without further experimental work these cells should not be used routinely.

(c) Incubation Period of Test Specimen with Antisera.

Considerable changes in sensitivity of the routine assay were occurring from day to day. This was finally traced to a variation in the

TABLE 3:7

EFFECT OF FREEZING AND THAWING OF ADSORBED SERA FOR F.D.P. ASSAY

Treatment of Test Sample	Serum Sample Number									
	1	2	3	4	5	6	7	8	9	10
	F.D.P. μ gm./ml.									
A. Fresh Sera, Adsorbed	3.2	3.2	3.2	6.5	4.3	7.8	7.8	10.4	20.8	10.4
B. Fresh Frozen Sera, Thawed and Adsorbed	4.3	3.2	4.3	6.5	4.3	5.6	5.2	7.8	20.8	10.5
C. Fresh Frozen Sera, Thawed, Adsorbed, Frozen and Thawed	3.2	3.2	4.3	6.5	4.3	7.8	7.8	10.4	20.8	10.4

time the test specimen was being left in contact with the antisera before addition of the sensitised cells, i.e. the contact time of antigen/antisera was varying between 1-4 hours. Experiments were conducted to ascertain the effect of varying periods of incubation upon the sensitivity of the assay.

Fig. 3:4 illustrates the results of one such experiment. With increasing incubation time, the end points of inhibition of both sera and diluted fibrinogen standard, moved to a higher dilution point, with the effect of increasing the calculated sensitivity of the system. Most of this change occurred within the first 6 hours of incubation, but a further change also occurred up to 24 hours.

The sensitivity of one batch of routinely prepared cells, with an incubation period of 30 minutes, was $2.2 \mu\text{gms/ml}$. However, with a 24 hour antigen/antisera reaction period, the sensitivity was decreased to between $0.3 \mu\text{gms/ml}$. and $0.5 \mu\text{gms/ml}$. Thus a method of greatly increasing the precision and discrimination of the assay was available which could be of considerable value in physiological studies. This method of increasing the sensitivity of a haemagglutination system is not new and has been described in the assay of insulin (Hales and Randle, 1963).

As a routine procedure, antigen and antisera were incubated together for exactly 24 hours before addition of the cells. Where great sensitivity was not required, the incubation period was reduced to 4 hours.

This change in the procedure greatly improved the routine reproducibility of the assay. Using this method, it was possible to demonstrate F.D.Ps. in all normal sera investigated.

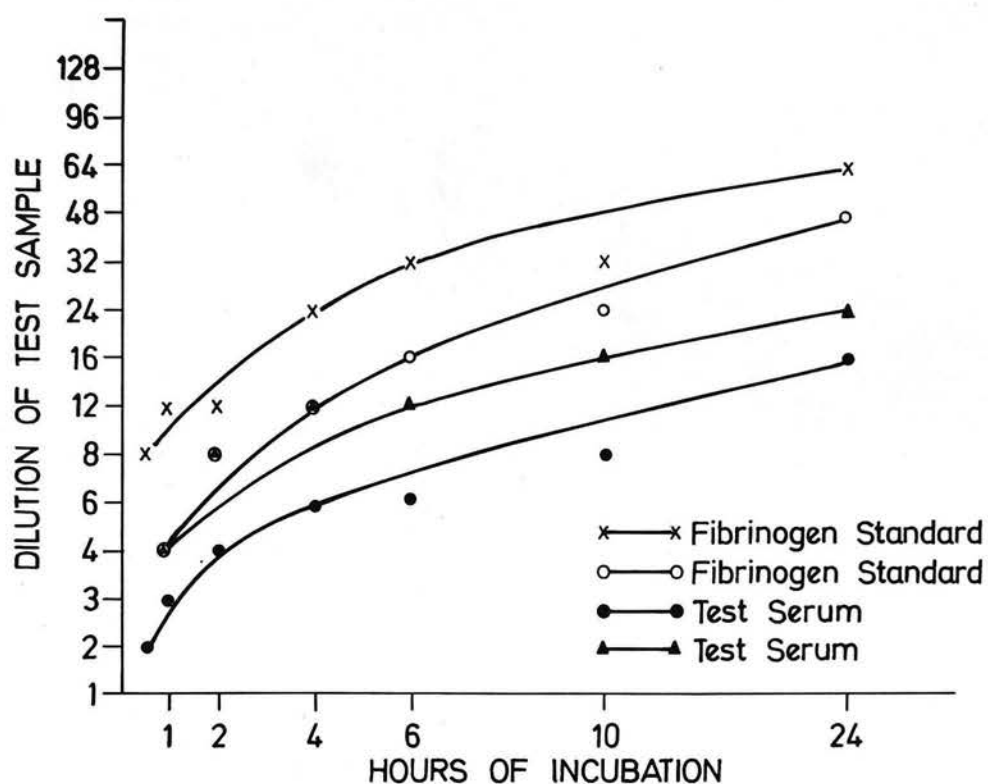


Fig. 3:4. Effect of increasing incubation time of the antigen/antisera reaction, in the T.R.C.H.I.I. assay, on the inhibition end points.

(d) Standards and Sensitivity of the T.R.C.H.I.I.

Initially, only one fibrinogen standard was used in the assay. As there are numerous technical errors in the preparation of dilutions of this standard, it was considered that the inclusion of three separate fibrinogen standards would reduce this source of error. The dilutions of these standards were so arranged that dilution steps near the expected inhibition end point were close together. The mean sensitivity was obtained from the three results. The sensitivity of a batch of cells, as used in this method, demonstrated only a small variation. In 27 separate assays over period of four months the mean sensitivities with standard deviations using dilutions of antisera of 1:5,000 and 1:10,000 respectively was 0.57 ± 0.16 and $0.45 \pm 0.14 \mu\text{gms/ml.}$ respectively. There was thus no great variation, over this time period, of the sensitivity of the cells, providing the incubation time of antigen/antisera was kept constant. Variations in sensitivity between days was also small.

As an additional check of the assay system a frozen standard adsorbed serum, collected from a normal donor, was included with each run. The end point of this serum, between assays, was remarkably consistent, with a slightly lower standard deviation than that of the fibrinogen standards. This could be attributed to the extra technical handling stages required in preparation of the standard fibrinogens. The mean value and standard deviation of this serum in 59 separate estimations over a period of 6 months was $6.4 \pm 1.3 \mu\text{gms/ml.}$

Using this system of controls, technical errors could easily be detected and assays in which there was marked variations from the expected

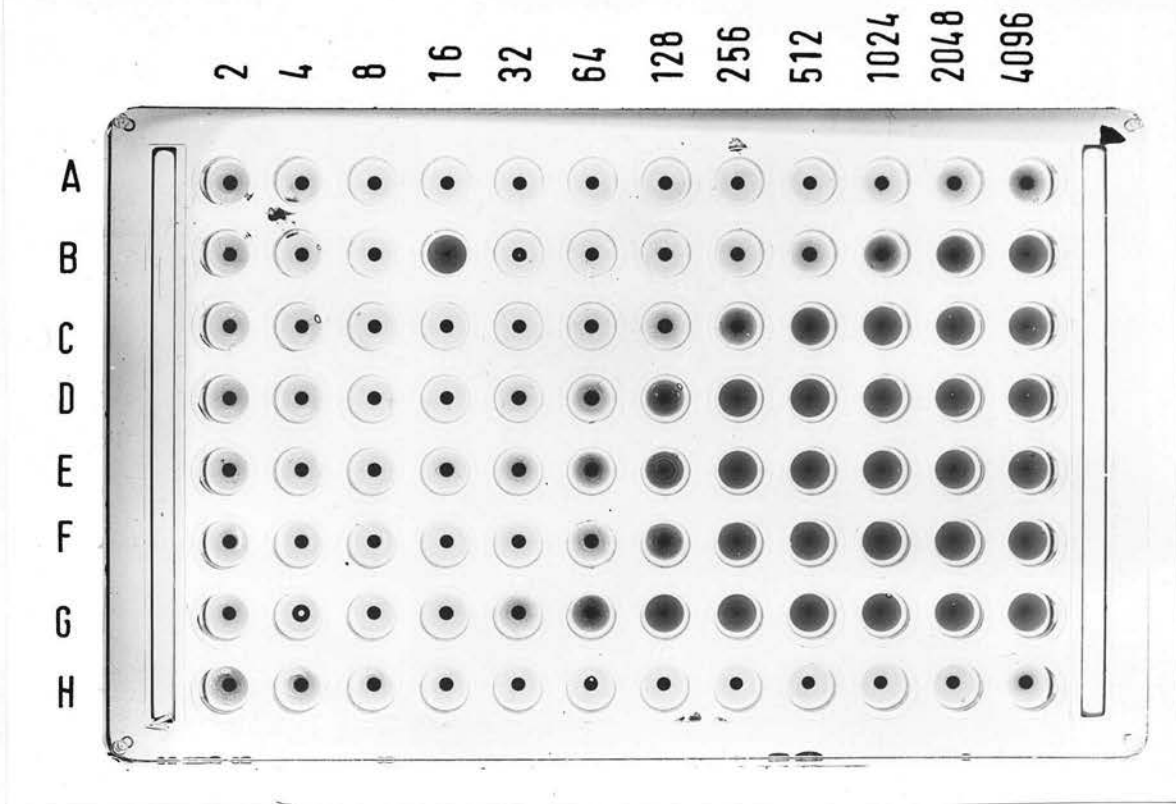


Fig. 3:4a. Photograph of a Microtiter plate used in a routine T.R.C.H.I.I. Assay.

The numbers at the top of the plate refer to the dilution of the test specimen. Letters to the side refer to test samples.

The inhibition end points can clearly be seen. Samples A-G represent sequential specimens from a patient with a massive episode of defibrination.

sensitivity were repeated. The assay as outlined, gave consistent results when these procedures were strictly adhered to.

5. Fibrinogen/Fibrin Degradation Products Assay (II).

A rapid, accurate and reliable test for fibrin/fibrinogen degradation products would be of considerable use in clinical work. Although the T.R.C.H.I.I. assay, as routinely performed, has proved a useful research tool it has the disadvantage of requiring 24 hours before results can be obtained. An investigation was therefore made of supplementary methods which might prove suitable for the rapid measurement of F.D.Ps.

A. Rapid T.R.C.H.I.I. Assay Method.

The method, as routinely performed, required at least 24 hours before results were available. However, if high sensitivity of the test system is not required an estimate of the F.D.P. level could be made within 2-3 hours of receiving the serum specimen. The modified method was as follows :-

- (a) Serum specimen reclothed with thrombin as per routine method.

Adsorption stage omitted.

- (b) Dilution steps performed as usual and fibrinogen standard set up in microtiter plate.

- (c) Incubation period of test specimen and antisera omitted and sensitised red cells added immediately.

- (d) Incubation at 4°C was for 2-3 hours. Non-agglutinated cells at the end of this time had usually settled, forming end points that could be read, and a preliminary estimate of the F.D.P. level obtained.

The plates were then returned to the refrigerator and final reading

made after 24 hours. The sensitivity of this system was as low as 5 μ gms/ml., depending on the cell batch, but this was not of critical importance when dealing with pathological samples.

Alternatively, the dilutions could be made up in plastic tubes and all reagents then mixed in similar tubes. The end point could then be read after 1 hour at 4°C.

It was thus possible to obtain rapid results using the modified routine T.R.C.H.I.I. assay.

B. Micro Haemagglutination - Inhibition Assay.

Israel et al. (1968) described a micromodification of the T.R.C.H.I.I. assay. This method apparently had the advantage of being a rapid test and of utilising a well recognised technique for detection of haemagglutination reactions in serological work (Chown, 1944; Chown and Lewis, 1946). This assay was therefore adapted for routine clinical use in the laboratory.

It was clear at the onset that the reagents were similar to those of the routine T.R.C.H.I.I. assay, apart from the concentration of sensitised cells and antisera. Therefore, for these tests the reagents were prepared as for the routine laboratory T.R.C.H.I.I. assay with the following modifications.

(a) Fibrinogen coated tanned red cells.

These were stored at 4°C, as already described, in a 2.5% solution. For use, the cells were reconstituted in one-third of the original volume of diluting fluid, making a 7.5% cell suspension.

(b) Antisera. (Hoechst Pharmaceuticals Ltd.)

This was used fresh at a dilution of 1:500 in diluting fluid, and kept at 4°C during the assay procedures.

The only other specialised equipment required was :-

Glass capillary tubes. Length 10 cm., internal diameter 0.1 cm. These tubes were marked with a felt writer at 5.5 and 6.0 cms. from one end.

Viewing Box. A box was constructed, surmounted by a sheet of white translucent glass mounted at an angle of 52° from the horizontal, and illuminated from behind by a white light source.

Plasticine. This was used for embedding the capillary tubes against the glass viewing box.

Glass microscope slides. These were used for mixing reagents.

The performance of the test was as follows :-

- (i) Antisera was diluted and kept at 4°C. Sensitised fibrinogen coated cells were reconstituted to a 7.5% suspension. A frozen fibrinogen standard solution was thawed and serial dilutions made.
- (ii) Test specimens were treated with thrombin, as previously described for the T.R.C.H.I.I. assay.
- (iii) Serial dilutions of the non-adsorbed test sera were made in diluting fluid in plastic tubes.
- (iv) Each sample was drawn up in the capillary tube to the 5.5 cm. mark, and this being followed by 0.5 cm. of diluted antisera, drawn up to the 6.0 cm. mark. The contents were then blown on to a glass slide, mixed for 5-10 seconds with the end of the capillary tube

and the mixture drawn by capillary action up into the tube again. Sensitised cells were then drawn up the capillary tube for a distance of approximately 0.5 cm. and the tube quickly inverted and the distal end embedded in plasticine against the illuminated glass. Haemagglutination was apparent within 20 minutes and tended to appear quite rapidly. End points of haemagglutination could easily be read without the aid of a magnifying glass and remained stable for several hours after the completion of the test.

- (v) The sensitivity of the system could be calculated from the fibrinogen standard, in a similar manner to the T.R.C.H.I.I. assay. The mean sensitivity in 28 separate assays was $7.0 \mu\text{g}/\text{ml}$. and there was only minor variations in sensitivity between days.

This method proved reliable and comparable results were obtained on the same sera in the T.R.C.H.I.I. assay (Fig. 3:5). A result was available within 30 minutes of receiving the thrombin clotted serum, and an estimation of whether the serum was "low" or "high" in F.D.Ps. could be made even sooner. The system appeared as stable as that of the T.R.C.H.I.I., and non-specific agglutination was not a problem in the specimens examined, even though preliminary adsorption of the sera was not performed. However, care was still required in interpretation of the results as a very occasional non-adsorbed sera assayed by the T.R.C.H.I.I. were found to have a strong non-specific haemagglutinin present that required dilutions of up to 1:32 before the effect disappeared. Such sera could be assessed by this method as having very low levels of F.D.Ps. unless dilution steps were continued to at least 1:128.

Technical manipulations of the assay were not difficult, and the

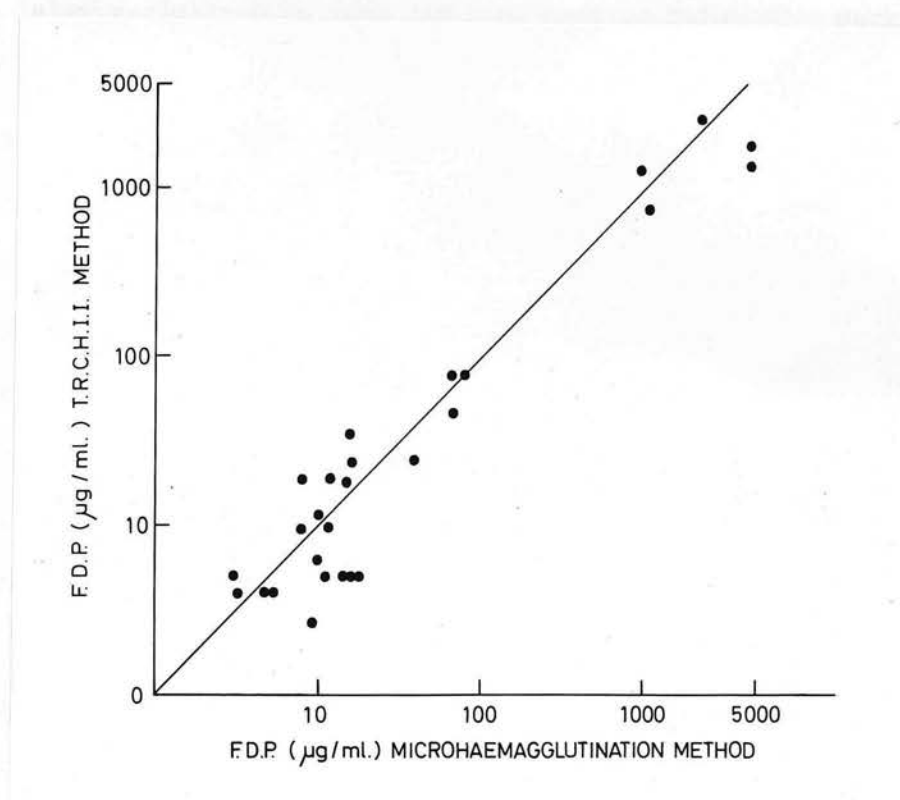


Fig. 3:5. Correlation between routine T.R.C.H.I.I. and microhaemagglutination assay systems for F.D.P.

quick assay method could be suitable for a routine coagulation-fibrinolytic laboratory. Sensitised cells suitable for the assay are now available commercially (Burroughs Wellcome & Co.)

C. Latex Tests.

A latex agglutination test has been used in laboratory work for the detection of rheumatoid factor (Singer et al., 1960). Latex particles coated with antisera, agglutinate in the presence of their respective antigen, and this method theoretically provides a rapid method of assessing the presence of specific antigenic material.

Commercial reagents, such as "Fi Latex" (Hyland Laboratories Ltd.), are available in kits for the detection of afibrinogenaemia. These fibrinogen antisera coated latex particles have also been utilized for the detection of serum F.D.Ps. (Merskey et al., 1966). A preliminary investigation was therefore undertaken of the value of latex coated particles in the detection of F.D.P. in serum.

Two types of latex coated particles were studied :-

1. "Fi Test" (Hyland Laboratories) Latex.
2. Latex particles (Burroughs Wellcome & Co.) coated with fibrinogen antisera (Hoechst Pharmaceuticals Ltd.)

Method:

Frozen serum samples were mixed with the latex particles on a glass microscope slide and mixed by rocking. The presence or absence of agglutination was observed over a period of 15 minutes.

The results in two representative patients are summarised in

Table 3:8.

It is evident that both false positive and negative results are occurring. In patient 1, in the first two samples, F.D.P. values are elevated, but the Latex Test was only a weak positive after a prolonged time. Positive tests were also obtained when F.D.Ps., as assessed by the T.R.C.H.I.I., were below 60 gm/ml. In patient 2, positive tests are slow to appear and in the initial samples, when F.D.P. values were high, are negative.

From these preliminary results, it was apparent that the Latex Test, at this stage of development was not suitable as a consistently reliable method for the detection of F.D.P. in fibrinolytic states. These findings are similar to Merskey et al. (1966), who also found false positive and negative reactions.

TABLE 3:8

USE OF THE LATEX TEST IN THE DETECTION OF SERUM F.D.P.
RESULTS IN SEVENTEEN SAMPLES FROM TWO PATIENTS.

Patient 1				
F.D.P. gm/ml. (TRCHII Assay.)	"Fi" Latex**		"B.W." Latex**	
	+ or -	Mins.	+ or -	Mins*
1572	+	15+	+	15
1290	+	15+	+	8
1894	+	15+	+	4
1125	+	15+	+	1
643	+	3	+	3
308	+	1	+	$\frac{3}{4}$
60	+	1	+	$\frac{3}{4}$
30	+	1	+	$\frac{3}{4}$
10	+	$\frac{3}{4}$	+	1

* Time taken for reaction to develop.

Patient 2.				
F.D.P. gm/ml. (TRCHII Assay)	"Fi" Latex**		"B.W." Latex**	
	+ or -	Mins.	+ or -	Mins*
1546	+	5	+	5
1843	-	-	-	-
772	-	-	-	-
722	+	10	+	10
774	+	10	+	9
193	+	3	+	4
48	+	5	+	10
5.1			+	11

** Abbreviations

"Fi" Latex - Hyland Laboratories.

"B.W." Latex - Burroughs Wellcome Ltd.

CHAPTER IV.

END POINT RECORDING DEVICES IN COAGULATION WORK.

Numerous instruments have been designed to measure automatically the latter phases of coagulation (Koffman, 1910; Dale and Laidlaw, 1911; Cannon and Mendenhall, 1914; Kugelmass, 1923; Dlias, 1931; Nygaard, 1941; Hartert, 1948; Barker, 1948; Elliot, 1952; Schnitger and Gross, 1954; Bishop et al., 1957). Very few have had popular appeal, and most laboratories to-day still rely on visual observations of fibrin end points by a skilled technician. Many of the end point recorders built for coagulation studies have been of complicated design and susceptible to mechanical failure, but recent instruments now becoming available appear to fulfil many of the necessary requirements for routine coagulation work.

Convincing arguments for the use of automatic coagulation end point recorders include the ability to standardise the measurement of the final phase of clotting, and a reduction of technical error and observer bias. Skilled as a technician may be, errors in assessing the end point of coagulation will always occur and will vary both within and between days. Observer bias and fatigue have always been problems in paired observations, and a minimisation of these factors by an objective end point recorder should considerably aid in improving the consistency and accuracy of coagulation results.

However, such a machine to record coagulation end points must be easy to operate (for even relatively unskilled personnel), achieve reproducible clotting times, have the capacity to deal with at least four clotting mixtures

at a time, have a low incidence of mechanical failure, and be versatile enough to deal with a wide variety of fibrin clot end points.

Several coagulation end point recording machines were investigated for possible use in these studies.

1. "Prothrombin Meter". (Evans Electroselenium Ltd.)

This instrument, which is based on a photoelectric principle, measures the change in optical density occurring as fibrin forms in a recalcified coagulation mixture. At a preset critical opacity, the circuitry operates to stop a previously activated pulse counter.

The two-channel machine studied was of basically good design and laboratory evaluation revealed that reproducible clotting times were easily recorded. Its use was limited, however, by poor temperature control, the small number of operating channels, the degree of adjustment of the controls needed before each clotting cycle, the restriction of the volume of clotting mixture required, and the poor ergonomics of the machine. In spite of the drawbacks this instrument has been utilized successfully for routine coagulation work by Eastham (1966).

2. "Fibrometer" Coagulation Timer. (B.D. Laboratories Inc.)

This machine operates on a different principle. Two electrodes, one stationary, the other moving, are introduced into a clotting mixture. Formation of fibrin is detected by the moving electrode, this then completes an electrical circuit and the machine is automatically stopped. The clotting time can be read from the counter mechanism to the nearest 0.1 second.

The basic design of this machine was very good. Because of transistorised circuits, it was compact and could be linked up to identical Fibrometer units. Reproducibility was satisfactory once experience was obtained with manipulation of the electrodes. It was, however, only a single-channel instrument so that only one clotting mixture could be tested at a time. Electrode cleaning was not always easy and thrombin contamination of the electrodes could easily occur if exceptional care in cleaning was not taken. The digital read-out counter, although calibrated in tenths of a second, was accurate only to 0.5 second, this being the time taken for the electrode arm to complete a full sweep in the clotting mixture. The machine is, at present, very expensive and to build up a minimum set of four units would be prohibitive in cost.

3. "Depex" Coagulometer. (Depex Ltd., de Bilt, The Netherlands).

This machine resembles in principle, the "Fibrometer". It is designed upon the plans outlined by Schnitger (1954), but has several advantages in comparison with the Fibrometer. These include multiple measuring channels, a superior electrode cleaning system, a minimum of adjustment procedures, and a much lower unit cost.

The instrument includes a heating block, into which clotting tubes containing incubation mixtures can be placed. Two electrodes are then introduced into the clotting mixture; upon the formation of a fibrin thread across the electrodes, an electrical circuit is completed; this in turn stops the counting mechanism previously set in motion. This machine was the most satisfactory of those tested and was selected for routine use.

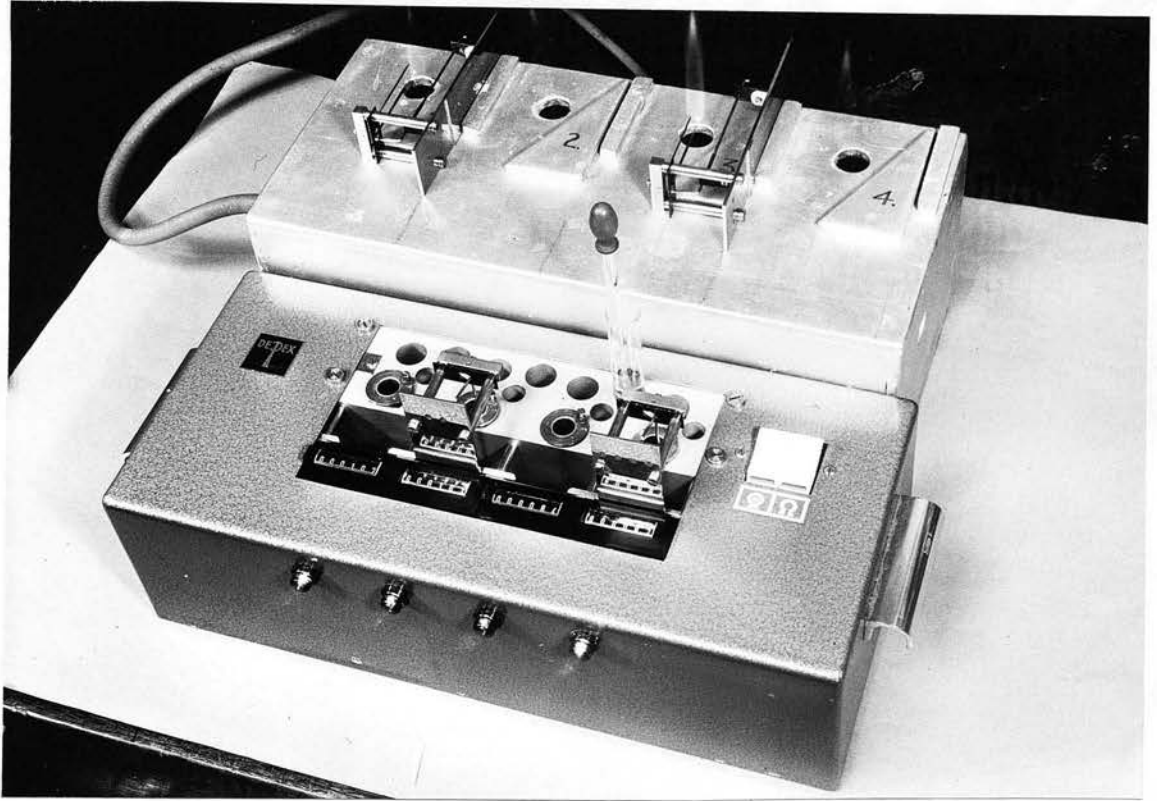


Fig. 3:6. The Depex Coagulometer.

The stand for cleaning the electrode is situated
behind the machine.

A. Description of the Coagulometer.

Fig. 3:6 illustrates the general design of the machine. The aluminium block is maintained at 37°C by a thermostat, and acts as the heating block for incubating mixtures. This heating block can be left on throughout a day's work. The machine has four channels, each with a separate counting mechanism activated by a press button type tumbler switch. Completion of a clotting cycle by the formation of a fibrin, is signalled by a small red light on the front of the machine and, at the same time, the counter automatically stops. The clotting time can then be read in seconds.

Fig. 3:7 shows in diagrammatic form the details of the electrode system. Two electrodes, one fixed and grounded, the other moving and isolated, are fixed to a metal holder. This is then inserted into the main machine housing and the counter mechanism started. When the electrode holder is placed into the machine, a revolving cam forces the moving electrode, consisting of stainless steel hook, in and out of the liquid once a second. When the movable electrode reaches its highest position, it is automatically connected with the grid of an anode tube provided with a negative tension of 24 volts via a resistance of 5 megohms. When clotting occurs, the hook pulls up a fibrin thread forming a low resistance connection between the moving and stationary electrodes, thus grounding the grid of the tube and causing an anode current which in turn activates a relay to stop the counter (Fig.3:8).

The electrode housing can then be removed from the machine, the electrode wiped with lint or a small brush, and heated in a non-luminous gas flame. After cooling, the electrodes are then ready for re-use.

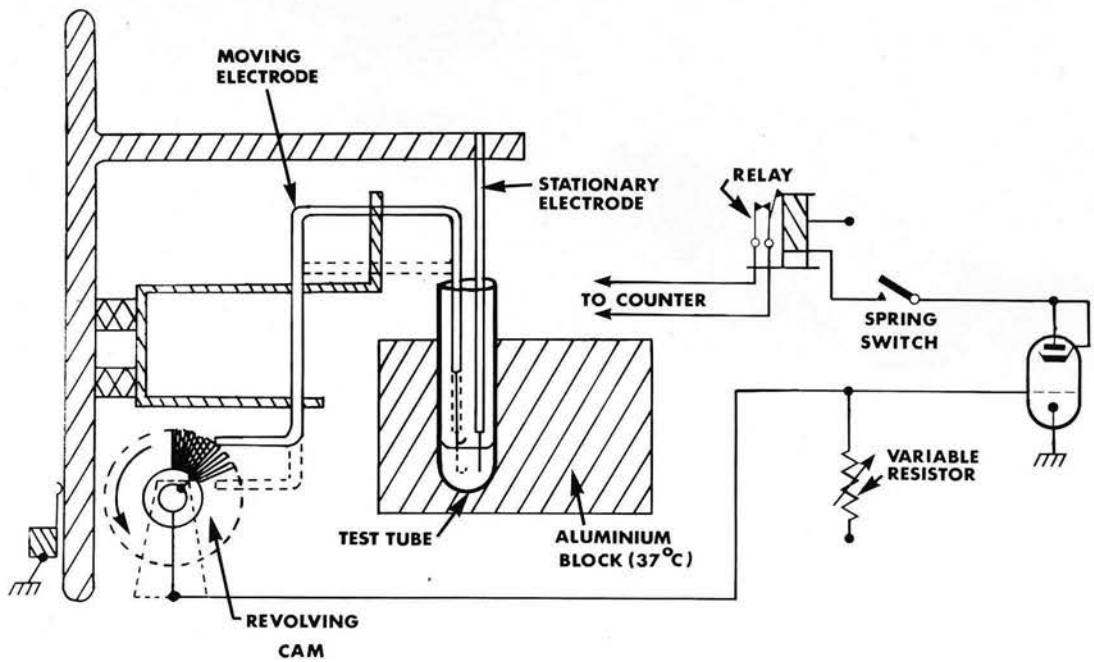


Fig. 3:7. Diagram of coagulometer electrode systems and circuit.

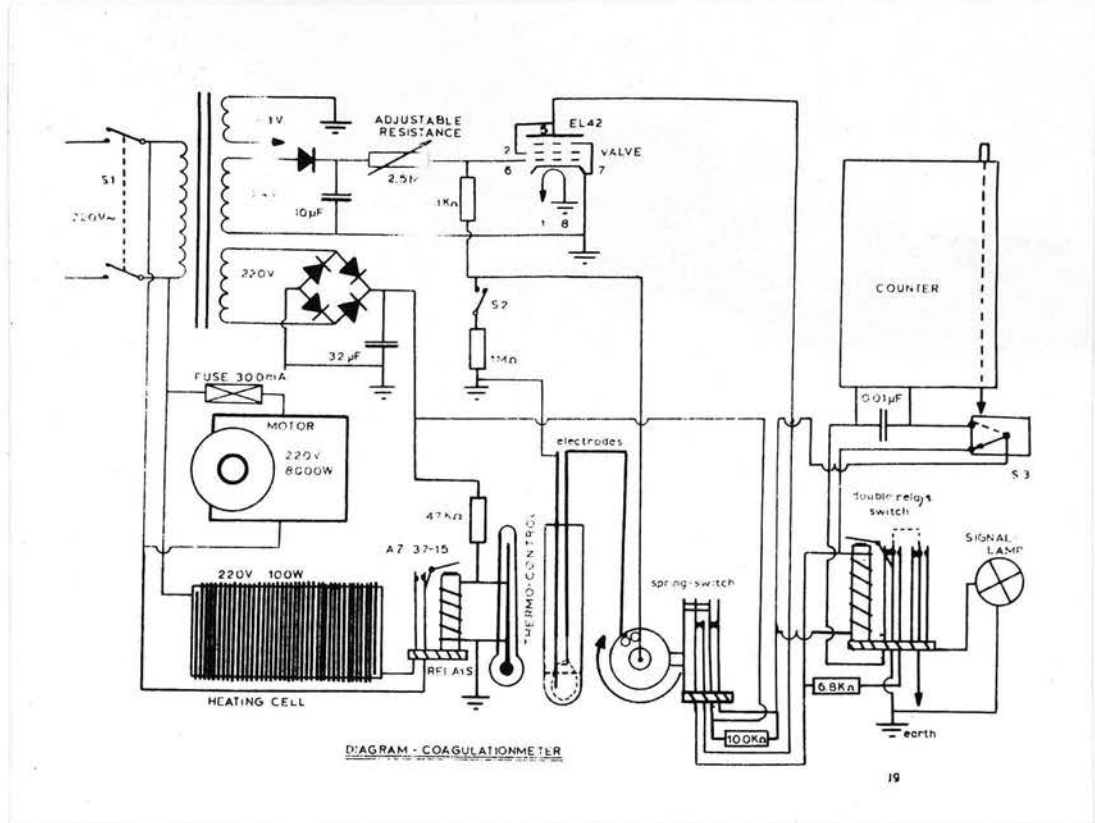


Fig. 3:8. Circuit diagram of Coagulometer.

B. Practical use of the Coagulometer.

The following factors were found to be important in the use of the Coagulometer.

- (a) 15 minutes was required for the heating block to reach a steady working temperature.
- (b) It was necessary for the electrodes to be clean and straight, and care was required in their handling.
- (c) The height of the tube holder had to be adjusted carefully to the volume of the incubation mixture (0.2 - 0.6 ml.)
- (d) The clotting tubes required insertion to their full depth into the tube holders; failing this the counter mechanism would automatically stop when the electrodes were added.
- (e) The electrode holder had to be placed completely into its grooved platform. If this was not done the counter would stop.
- (f) Only one size of tube could be used (52.5 x 11.5 mm.). Major variants in tube size were unsuitable for the machine.

Disturbances in the use of the Coagulometer were relatively few, but the following were noted :-

- (a) The heating of the aluminium block was variable. Temperatures throughout a day varied between 36°C and 38°C in one machine and 35°C and 38°C in the other. A built-in visual thermometer was not supplied and checks were frequently made of the temperature. On one occasion when the thermostat failed, the temperature rose to 56°C without warning and several experiments were invalidated. This aspect of the machine could be improved.

- (b) After considerable use, the camshaft of the movable electrodes showed signs of wear, and jamming of the revolving shaft occurred. This was rectified by replacing the electrode, but this required considerable adjustment of the electrode housing before reproducible results could again be obtained.
- (c) The pulse counters could not always be returned to true zero. This fault was inherent in the design of the machine and can introduce random error of up to one second in a determination. The position of the camshaft determines whether the counter can be returned to zero.
- (d) Irregularities of the clotting time could usually be traced to faulty techniques or variations in test tube shape. The machine was otherwise trouble free, although reasonable care was required in performing tests, otherwise poor results were obtained.

C. Reproducibility Experiments.

Prior to use of the machine in these studies, an investigation of its reproducibility was made. The kaolin-cephalin clotting time measurement was used as a test method.

Fresh plasmas were obtained and maintained at $+4^{\circ}\text{C}$. At two minute intervals eight tubes containing 0.1 ml. plasma and 0.2 ml. kaolin-cephalin reagent were introduced into the heating blocks of two machines and allowed to incubate for 10 minutes. At the end of this time the tubes were recalcified with 0.1 ml. of 0.025 M calcium chloride and the clotting time recorded. Thus six clotting times at each of eight measuring points were

obtained, a total of 48 observations on each plasma sample. A typical set of results is recorded in Table 3:9.

TABLE 3:9

THE KAOLIN-CEPHALIN CLOTTING TIME PERFORMED ON THE SAME PLASMA USING TWO COAGULOMETERS IN SERIES. MEAN VALUE 39.1 ± 0.9 SECONDS

Machine 1				Machine 2			
1	2	3	4	1	2	3	4
Seconds				Seconds			
40	40	41	41	-	40	40	39
39	39	40	39	39	38	38	39
39	39	41	40	40	40	39	39
36	38	40	39	39	39	39	39
39	38	39	40	40	39	39	38
38	39	39	40	38	39	39	38
Mean							
38.5	38.8	40.0	39.8	39.2	39.2	39.0	38.7

In three separate such experiments on different plasmas, the mean clotting times with standard deviations were :-

Plasma A. 40.2 \pm 1.3 seconds

Plasma B. 39.1 \pm 0.9 seconds

Plasma C. 50.0 \pm 1.2 seconds

Mean standard deviation = \pm 1.1 seconds

There was no significant difference between the clotting times obtained with separate machines (Plasma A: $t = 1.079$, $0.2 < p < 0.3$; Plasma B: $t = 1.076$, $0.2 < p < 0.3$ N.S.; Plasma C: $t = 0.711$, $0.5 < p < 0.4$ N.S.). There was, however, a significant difference between channels, but this varied between experiments, and no single channel was consistently recording significantly higher (or lower) results.

In Experiment A there was a significant difference between channels 1 and 3 in Machine 1 ($0.05 < p < 0.025$) and channels 1 and 4 ($0.1 < p < 0.05$) in Machine 2, but in Experiment B there was a significant difference between channels 2 and 3 ($0.02 < p < 0.01$) in Machine 1, and in Experiment C between channels 1 and 4 ($0.1 < p < 0.05$) in Machine 2. These results indicated a random error and could be explained by random variations in technique, e.g. not recalcifying at exactly the same time in each tube, and delay in pressing the activating counter.

Other experiments were performed to assess if the time at which the electrode was inserted into the clotting mixture affected the clotting time. Plasmas, previously activated with kaolin-cephalin reagent were recalcified and the electrodes introduced at varying intervals, i.e. immediately after recalcification, 15, 30 and 45 seconds later, and prior to

the known coagulation time of that plasma. The results consistently demonstrated that the time of electrode introduction made no difference to the resultant clotting time. However, in most assay systems described, the electrodes were introduced with 20 seconds of recalcification.

The effect of carbon collections on the electrodes was also investigated. After each clotting test, the electrodes were wiped with lint or a brush and flamed in a gas flame. Carbon commonly formed on the electrodes if these were not properly cleaned. Several experiments showed that if the electrodes were not well flamed irregularities of the clotting time were more common, presumably due to carry-over of contaminating thrombin or thromboplastic material.

There was a good correlation between assays performed on the same plasmas by both visual and machine techniques ($r = 0.888, p < 0.001$) in a small series of twelve separate plasmas. With experience the machine technique was found to be less tiring and multiple samples could be handled easily. Clotting times generally improved when the technician had become thoroughly acquainted with the machine and was not disturbed while running multiple sets of assays.

D. Comment.

On the basis of these tests and practical routine experience the machine was accepted as a reliable instrument with the provision that determinations of clotting time in a single plasma sample required measuring at more than one recording point. Therefore, determinations of clotting time were always carried out in duplicate and often in triplicate.

The coagulometer was not used for determinations of clotting time below 20-30 seconds, e.g. as in the thrombin, prothrombin times, and two-stage assays based upon the thromboplastin generation test. The "resolving" power of the moving probe is ± 1 second, and thus the percentage error at such short clotting times would be higher. Satisfactory results could be obtained visually. However, if short clotting times were done in triplicate this criticism was only partly valid. It was also found that very long clotting times, with slow formation of fibrin (as with dilute solutions of thrombin and of fibrinogen) were difficult to record with the coagulometer and this type of infrequently occurring gel end point, i.e. greater than 300 seconds, was usually performed visually.

Experience with the machine also demonstrated that different operators would obtain closely similar coagulation times on the same plasma sample. Once routines were established technician fatigue was greatly reduced, and many more assays could be handled with greater accuracy.

In summary, the Depex Coagulometer fulfilled many of the criteria for a practical laboratory end point recorder. Its accuracy, multiple channel design, ruggedness, and simplicity were well substantiated. The main disadvantage was the inconvenience of the electrode cleaning method, but this could be minimised by the construction of a suitable electrode stand. Most of the work described in this thesis was based upon the practical use of this instrument. Such a machine could easily be integrated into a routine coagulation laboratory service and the use of several machines could greatly increase the output of accurate and consistent coagulation work.

CHAPTER V.

COAGULATION AND THE COMPUTER.

The bioassay of coagulation factors is a well recognised facet of the work in many routine haematology laboratories. Such assay work is often both time consuming, technically demanding, and is susceptible to varied errors. For these reasons, all aids to eliminate avoidable error are worthwhile, particularly if reproducibility of technique can be improved.

One possible source of error in coagulation assays lies in the calculating of the final results. Most assay systems require the plotting of parallel dose-response curves by eye on suitable logarithmic paper and estimating the horizontal shift necessary to superimpose one line on the other. This measurement is then proportional to the potency of the unknown sample, and for most purposes, this type of graphical calculation is both quick, simple and satisfactory. However, with some results, drawing the line of "best fit" may be difficult, and graphical calculation may obscure the fact that the test and control results are lying on dissimilar portions of the dose-response curve. Also, graphing of results is not easy when clotting times overlap. Another appreciable source of error is the variation between observers when plotting identical sets of results.

For these reasons computed results, based upon the information obtained in assays, would be mathematically sound and would reduce a source of technical error. Such calculations are now available but are complex and unsuitable for routine use, except in special circumstances (Finney, 1952; Savage, 1959; Hardisty and Macpherson, 1962; Baumgarten et al., 1963;

Ingram, 1965).

A study was therefore made to assess if a suitable programme, which would routinely compute coagulation assay results, could be written for the Edinburgh University K.D.F.-9 Computer.

With the co-operation of Mr. W. Lutz, Edinburgh University Department of Statistics, a programme was written in Atlas Autocode based upon the mathematical information supplied by Ingram (1965) and Hardisty and Macpherson (1962). The mathematics on which the programme were evolved are included in the appendix, statistical methods (page 134). The programme included not only a computation of the potency of the test as compared with the control sample, but also a calculation of the 95% confidence limits of the assay.

1. Principles of the Computer Programme.

The basic principles were as follows, and each stage may be identified in Fig. 3:9. (page 134).

Step 1.

Regression equations were calculated for both the test and control plasma clotting times (the dose response lines).

An analysis of variants ("F test") was then performed to test for linearity of the calculated lines. If either line was not linear, the value for F was automatically printed as well as the critical F value at the 5% significance level. Suitable F test tables were included in the initial programming.

Although the test for linearity was not strictly necessary at this

stage of calculation (Hills, 1968), it was found useful as an additional check for gross errors in clotting time results, or mistakes in teletyping.

Step 2.

The dose response curves were further mathematically tested, in two steps, for any departure from parallelism.

(a) Test for homogeneity of variance.

This test was included as a check that it was reasonable to pool the variances of the two calculated lines, before proceeding with the next stage, i.e. application of a significance test (t test) for parallelism.

Only very large variances, i.e. 5 - 1 affect the application of this form of t test, and again the test is not strictly necessary. However, it was included as an additional check for gross discrepancies in variance. A suitable F test table was included in the programming, and again the value for the pooled variances (F) was printed out, and if this was significant the critical F value at the 5% significance level also printed.

(b) Application of the t test.

This computation is designed to assess if the regression lines are significantly non-parallel. Biological assays as used in coagulation work do not always yield strictly parallel lines, and allowance must be made for this in assessing departures from parallelism. It is considered that if non-parallelism was apparent at the 5% level of significance, the initial clotting results should be reassessed for technical error.

The programming was so arranged that if the regression lines were significantly non-parallel (5% level), the t test result would be printed out along with the critical t test at the 5% level of significance,

and the words "NON-PARALLEL".

Whether the lines were parallel or not the computer would automatically proceed to the next stage.

Step 3. Calculation of Common Slopes and Potency Ratio.

If the dose response curves were not significantly non-parallel the potency ratio (M) could be calculated from a formula which includes a value for the common slope of the two lines. The potency ratio is obtained from the anti-log of M , which can then be expressed as a percentage.

Both the log potency ratio and the potency ratio (percent) were printed out.

Step 4. Calculation of Standard Error and Confidence Limits of Potency Ratio.

The formulae for this calculation is included in the appendix. The standard error of both the log potency ratio and the potency ratio are calculated and printed out. The 95% confidence limits were obtained from t test table entries previously entered in the computer. From the standard error of the potency ratio, the standard error expressed as a percentage of the potency ratio could be obtained.

2. Programme Safeguards.

There were several safeguards included in the programme to detect discrepancies in either experimental technique or typed results. In brief these included :-

- (a) In the final print-out pattern (Fig. 3:9) the original clotting times of both test and control plasma are included, and this allowed quick reference against the original results.

CONTROL					
10	55.90	61.00			
20	65.00	66.20			
40	74.60	73.90			
80	77.20	77.90			
YC=B+X*CON					
-19.6160	40.9640				
TEST					
10	67.20	67.70			
20	74.50	75.30			
40	77.90	76.20			
80	81.50	82.90			
YT=B+X*CON					
-15.6765	52.5405				
VARS ABOUT REG LINES	VAR(CONTROL) =	2.134750	VAR(TEST) =	1.659333	
F =	1.2665		a) TEST FOR HOMOGENEITY OF VARIANCE		
T =	-1.9238	DF =	12	b) T TEST	
LOG POTENCY RATIO =	-0.3492				
SE OF LOG POT RATIO =	0.0340				
POTENCY RATIO (PERCENT) =	44.7513				
95 PERCENT LIH =	35.3575	10	56.6407		
PERCENT ERROR =	79.0069	TO	126.5679		

Fig. 3:9. Computer "print-out" sheet.

- (b) If the first F value was significant, the words "NON-LINEAR REGRESSION" were printed out. This would be a warning to reassess the initial clotting results.
- (c) If the second F test (homogeneity of variance) was significant this would be printed out. Values of F at this stage above 5.0 were usually significant and commonly denoted technical error.
- (d) The final safeguard was after the t test for parallelism. If parallelism was not present, this would be printed out with the words "NON-PARALLEL".

These were found to be satisfactory safeguards to protect against errors on the laboratory side. Many hundreds of assays, of varying combinations of results, were tested, and although care was required in the interpretation of the final "read out" sheet, anomalies were relatively few. With more experience of the method it might be possible to abbreviate the "read out" sheet further, perhaps by eliminating safeguards (b) and (c), although it was felt that these were useful as additional checks on procedure.

3. Comments and Results.

The programme was used extensively in calculating the results outlined in this thesis. It was found to be applicable to one-stage coagulation Factors VIII, IX, and XII assays as well as to two-stage Factor VIII assays. It could theoretically also be used for other assays (Factors V, VII, and X) although this was not routinely done as only relatively small numbers of these types of assays were performed.

In Fig. 3:10 are charted the results of both graphical and computer calculations of test sample potencies. There is good agreement between the two sets of results.

It was noted that graphed calculations of the test sample potency, as determined by two separate observers, gave a mean absolute variation of 9.5%, i.e. on the same set of clotting results, different observers would obtain final potency values within 9.5% of each other, over a wide range of values. When each observer's results were compared with the results obtained from the computer, the mean absolute variation was 7.2%. One conclusion to be gathered from this information is that if different workers are graphing and calculating results within a laboratory, there is an inherent absolute error, probably as high as 10%, in estimating the final potency of the test sample. Although this may not be of great significance in most pathological conditions, in coagulation factor replacement work and in physiological studies, the error may be appreciable. The other conclusion to be gathered is that, on the average, the mathematically computed results and the graphically computed results will vary in absolute values by up to 7%. It can be appreciated that consistent results could best be obtained if one observer performed all the final calculations of test potencies; such a procedure is technically not always practical and thus mathematical computation of final results is preferable.

It was also found that the ready availability of the 95% confidence limits of the assay was particularly useful. Firstly, it could be seen at a glance if the confidence limits for any particular assay were within the known acceptable range for that assay, thus allowing the rapid detection of experimental

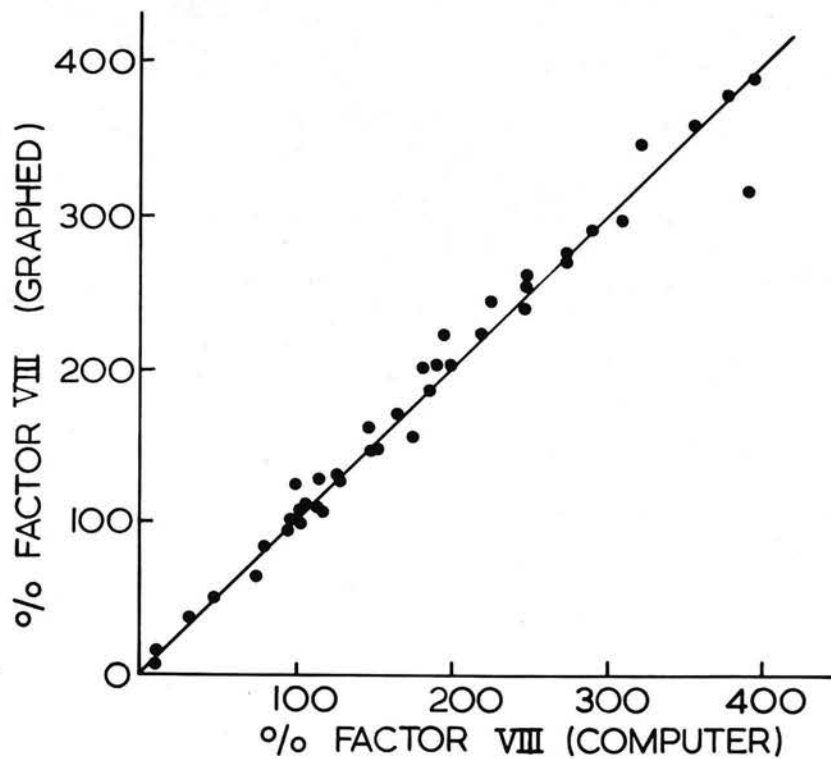


Fig. 3:10. Correlation of Factor VIII assay results as calculated by computer and graph.

or technical errors. Secondly, the confidence limits would improve with continued practice of the assay. These confidence limits could thus be used as a guide to the technical progress of laboratory staff. Thirdly, the effect of variations in technique could partially be assessed by the changes occurring in the confidence limits, as the formula on which the limits are based include not only the experimental errors of both the standard curve and test plasma results, as obtained in the routine use of the method, but the slope of the dose-response curve and the potency difference between the test and control plasmas.

There were thus numerous advantages in the use of the computer for calculating coagulation results. On the other hand, the process was time consuming. Not only had the results to be compiled carefully, but considerable time was required to teletype the results on to tape before programming the computer. Between 20-30 assays could be prepared in an afternoon's work, and there were numerous points at which script errors could occur. The final results were also not available rapidly. It is possible that a similar mathematical programme could be prepared for a desk-type computer, such as the Olivetti 101. Arrangements are in progress for this, and if this is practical, computation of coagulation results could become a rapid routine procedure in many coagulation laboratories. However, it is clear that the computation of coagulation results is a step towards automation of clotting techniques. If a semi-automatic clot timing machine could be constructed it should be possible to feed the clotting times directly into a teletyper linked to a computer, thus allowing the immediate return of results. Such an arrangement is at present being studied elsewhere (Denson, 1968).

There are only a few references in the literature to the use of computers in coagulation work. Veltkamp (1968) mentions use of a digital computer in the calculations of lines of best fit in coagulation work, and Grant and Biggs (1966) have used an Elliot 803 computer to compute analysis of six point parallel line assays. Ingram (1967) has also prepared a suitable programme.

It would appear that this method of computation of clotting results could be of considerable use in sequential research investigations or in departments which require consistent reproducibility of assay results, as in a Blood Products Unit.

If a rapid method of computation could be evolved, improved standardisation of assay techniques is a practical proposition.

CHAPTER VI.

STANDARD REFERENCE PLASMAS

In view of the known lability of coagulation Factors V and VIII on storage (Bidwell, 1955; Wolf, 1959; Macfarlane and Biggs, 1962; Britten and Grove-Rasmussen, 1966), the choice of a stable reference standard for use in assay studies on these factors presents problems. There are evidently great differences in the standards adopted between laboratories, and these variations, along with the inherent error in bioassay of coagulation factors, often make interpretation of reported results difficult. In the work described in this thesis it was thought necessary to investigate the relative value of different types of standards, as in the research investigation of physiological and pathological states of haemostasis it is of some importance to have a reliable reference standard, particularly for serial studies.

Much work has been directed to evolving a suitable standard preparation for Factor VIII (Grant and Biggs, 1966), but conclusive evidence for the superiority of any one preparation is still lacking. Preparations at present in use, or under investigation, include :-

- (a) A fresh frozen plasma, stored in small aliquots at -20°C or below and collected from 1-30 normal donors (Bergna, 1960; Preston and Barr, 1964; Abildgaard et al., 1967; Garner, 1967).
- (b) Lyophilised preparations made from human or animal plasma of concentrates (Baumgarten et al., 1963; Kekwick and Walton, 1964; Grant and Biggs, 1966; Simone et al., 1967).
- (c) The use of a selected donor whose levels of coagulation factors have

been assayed repeatedly (Pitney, 1956; Pool and Robinson, 1959; Edson et al., 1967; Veltkamp, 1968).

In this study, these three types of standard have been assessed.

1. Pooled Frozen Reference Plasma.

This was prepared as follows :-

10 ml. of blood were collected into prechilled plastic tubes from 12-18 normal blood donors at the conclusion of a donation of 400 ml. of blood. Care was taken to avoid excessive venous occlusion and the venepuncture was carefully performed. Preliminary experiments were made, comparing a single sample of blood separately collected from a post-blood donation sample, for the levels of Factor V, Factor VIII, the kaolin-cephalin clotting time and the prothrombin time. There was no significant difference between the pre and post donation results and it was therefore concluded that samples taken post donation were satisfactory for use in a control plasma pool. No selection by age was made. Samples were collected between 9.00 a.m. and 10.00 a.m. at a routine blood donor clinic. The blood samples were centrifuged at 3,400 r.p.m. (2,230 g) for 30 minutes at 4°C and later pooled in a siliconised container, also kept at 4°C.

Small aliquots of plasma from each donor sample were obtained and the kaolin-cephalin clotting time performed on each sample. Plasmas with clotting times lying outside one standard deviation from the known mean clotting time of this test system were not included in the main plasma pool. This procedure was in an attempt to screen out donors with either very high or low levels of plasma intrinsic clotting factors, as the inclusion of extreme

values of one or more clotting factors could produce a mean pool value of well over or under 100%.

After pooling of the selected plasmas, small aliquots (0.5-1.0 ml.) were dispensed into pre-cooled plastic tubes and immediately frozen in an open tube rack at -40°C . For use, the standard was thawed at 37°C with constant mixing to ensure complete solubility of any cryoprecipitate formed during thawing, and then used immediately.

Pools prepared in this way were of surprising uniformity; there were only small variations between Factor V and Factor VIII levels of separate pools when routinely assayed against each other.

For assay of Factors V and VIII, the pooled plasmas were not used for longer than three weeks after preparation. For other assays, storage was continued for up to two months, although in most cases the pooled standard was depleted in a much shorter period. However, it was not possible to be certain that all pools were exactly 100% of normal, as a stable standard for separate comparison between pools was not available over the complete time period.

Additional experiments were performed on the stability of Factor V and Factor VIII in these prepared standards.

(a) Factor V.

The standard curve for this assay was prepared at twice weekly intervals for six weeks. A standard pool of phenolised rabbit brain thromboplastin was used throughout the experiment and the same stored Factor V deficient plasma. The clotting times obtained are summarised in

Table 3:10. It can be seen that the curve is very constant for three weeks with only minor variations in clotting time. After this time, however, there is small but appreciable lengthening of the clotting time at most dilutions and this was attributed to deterioration in Factor V activity. Accordingly, pools of plasma were not used for longer than three weeks after preparation.

(b) Factor VIII.

A similar experiment was attempted with the Factor VIII assay throughout the period of study. The results were not conclusive as technical continuity was not possible. It was noted that variations occurred in the clotting times of the same dilutions of the standard plasma between days, but the slope of the graphed standard line did not alter appreciably. The explanation of this phenomena was possibly due to small alterations in technique and reagents. The clotting times of separate aliquots of the reference plasma gave virtually identical clotting times throughout a day's work, although there was, as mentioned, intra-day variations.

A procedure was arbitrarily adopted whereby control standard plasma for Factor VIII assays was not used for longer than three weeks after being collected. A new pool was then prepared.

Levels of Factor VIII may be well maintained at -40°C when the above collection procedure is followed (Biggs, 1964). In six separate Factor VIII assays on out-dated standard plasma standards, stored for up to three months, the Factor VIII levels lay between 85% and 100%, mean 93% of normal plasma.

This form of prepared standard was used throughout all studies,

TABLE 3:10

THE STANDARD CONTROL CURVE OF FACTOR V ASSAY PERFORMED
TWICE WEEKLY FOR SIX WEEKS ON A STANDARD POOLED PLASMA.

Dilution of Plasma		$\frac{1}{1000}$	$\frac{1}{200}$	$\frac{1}{100}$	$\frac{1}{20}$	$\frac{1}{10}$	B
(Clotting Time in Seconds)							
Week 1	A	82	54	46	31	25	93
	B	83	54	46	30	25	102
Week 2	A	88	54	46	30	25	96
	B	80	55	46	30	25	101
Week 3	A	88	53	48	31	26	92
	B	80	57	46	30	26	92
Week 4	A	75	60	49	32	28	100
	B	89	59	48	35	27	97
Week 5	A	97	60	49	36	28	106
	B	-	-	-	-	-	-
Week 6	A	90	62	51	36	29	103
		-	-	-	-	-	-

and was found to be a reliable preparation. The intra-batch reproducibility of the standard plasmas may have been related to the rigorous screening of samples before being included in the main pool, and to the low temperature storage. In support of this Edson et al. (1967) have demonstrated that high intrinsic coagulation factor levels are very commonly associated with short kaolin-cephalin clotting times.

2. Lypophilised Reference Plasma.

20 ml. of blood was collected by separate venepuncture by the usual technique from 20 normal donors (equal ratio males : females; mean age 36 years), centrifuged at 3,400 r.p.m. (2,230 g) for 30 minutes and pooled in a siliconised container. Using an accurately graded siliconised burette exactly 1.0 ml. of plasma was added to small siliconised glass tubes and these were immediately frozen at -70°C in an alcohol/solid carbon dioxide mixture. The plasma was then lyophilised by a careful technique, filled with nitrogen, sealed and stored at -40°C . For reconstitution, 1.0 ml. of distilled water was added and the solution used as soon as possible.

The results using this standard preparation are summarised in Fig. 3:11. At regular intervals the dried standard was compared with a pooled normal freshly prepared frozen standard by a two-stage Factor VIII assay technique. For four months, the dried standard gave results comparable to the different pooled plasmas, i.e. close to 100% normal. This was surprising as some loss of Factor VIII was thought to be inevitable on lyophilization. Although the drying process had been carefully controlled, it is probable that the initial pool value, before drying, was above 100% as

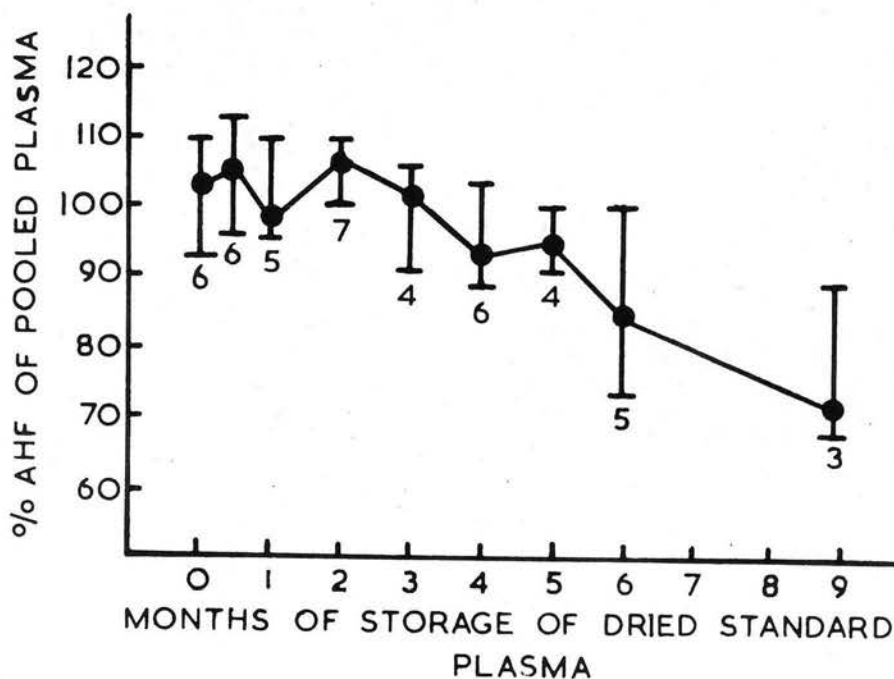


Fig. 3:11. Results of repeated assays on a freeze-dried standard plasma over a period of nine months.

The numbers beneath the graphed line refer to the number of assays performed at each test.

no screening for high Factor VIII levels of the samples had been performed before pooling was performed. The potency of this standard however, was assessed by another laboratory at 102% (mean of 18 assays) (Denson, 1966).

After four months the range of values obtained on assay of the standard was lower, and some tubes yielded consistently lower results. At six months the mean value of Factor VIII was 84%. At nine months the mean value was 72%. The deterioration in the lyophilised standard was also confirmed by the routine Blood Coagulation Service in separate assays in relation to Factor VIII concentrates. The standard was also again assessed by the Oxford Laboratory, and the Factor VIII level was now found to be 83% (Denson, 1967).

Possible factors contributing to the alteration in Factor VIII levels include :-

- (a) Sealing of some tubes had been unsatisfactory. Moisture is deleterious to Factor VIII stability in concentrates (Kekwick and Walton, 1964) and it is feasible that this factor alone could explain the change. In future preparation sealed ampoules would be more satisfactory, but these were not available at the time of the initial experiment.
- (b) Initial lyophilising may have been inadequate. The criteria of adequate drying has not been well defined for Factor VIII concentrates but the process in this department usually yield a moisture content of less than 0.1-0.5% of dry weight, which is comparable to other centres (Ménarche, 1967).
- (c) The temperature of storage of the standard, which was usually below

-25°C, had been inadvertently increased to between -5°C and -10°C for at least two weeks on one occasion. Low temperatures may be important in the preservation of Factor VIII in dried samples (Kekwick and Walton, 1964; Thelin, 1968).

- (d) Variations in the levels of Factor VIII in the eight different donor frozen plasma standard pools may have occurred.. This was minimised by the use of pools prepared as in Section 1. That these pools were not markedly different was evidenced by the small variations between pools when assayed against each other. However, this factor cannot be disregarded, and could explain the results obtained in the later months.
- (e) The basic assay system may have altered. There was no real evidence for this, as reagents and techniques had been standardised as far as was practical throughout the period. Nevertheless, the assay system is far from precise and this factor cannot be excluded. It is less likely in view of the results obtained from the Oxford Laboratory.

The final conclusion was that a lyophilised plasma reference standard could be prepared but considerable care would be required in its production and storage. These conclusions are similar to Thelin (1968) describing a commercially prepared Factor VIII standard (Hyland Laboratories).

3. Selected Donors for Standard Reference Plasmas.

This method of control can be applied to Factor VIII assays (Veltkamp, 1968). Freshly withdrawn plasma from selected donors are first assayed against a range of normal donors, and the mean values obtained. A

correction factor is then applied to convert the test sample value to a percentage of 100%. Alternatively, plasma from the same donor is withdrawn at fortnightly intervals and frozen in small aliquots. This method, although suitable if the donor is known to be exactly a 100% Factor VIII level, is not practical in a routine laboratory.

Control of Factor VIII assays by this method was not fully investigated. In a limited study, the two individuals studied appeared to show variations in Factor VIII levels when assayed against a frozen pooled plasma both within and between days. The frequent bleeding of the same donors was also regarded as being inconvenient. Some workers have reported that the Factor VIII levels of individual subjects are relatively constant (Grant and Biggs, 1966), but other recent workers utilising a standard lyophilised reference plasma (Simone et al., 1967) have not been able to confirm this. In view of the known fluctuations of Factor VIII with adrenaline or exercise stress, it would seem unwise to regard any individual as having a constant level of Factor VIII. Accordingly, this type of control method was not used; further investigation with stable standards would be helpful in assessing the degree of variation normally occurring in individuals.

Most of the above work refers to Factor V and Factor VIII assay systems as the problem of suitable controls is a greater problem with these less stable factors. Although the remaining coagulation factors are stable, it would be a major step forward if a suitable standard lyophilised preparation could be made available for use in the assay of these factors, thus reducing intra laboratory variation and allowing more critical comparisons of coagulation assay results.

CHAPTER 1.

THE EFFECT OF EXERCISE ON BLOOD COAGULATION AND FIBRINOLYSIS

Introduction.

The effect of muscular exercise on blood coagulation has been studied widely in both man and laboratory animals (Hartman, 1927; Mills and Necheles, 1928; Vouri, 1950; Schneider and Zangari, 1951; Warnock et al., 1957; Rizza, 1961; Keeney, 1962; Egeberg, 1963; Goudemand et al., 1964; Von Kaulla and Von Kaulla, 1964; Finkel and Cumming, 1965). Other workers have investigated the changes in the fibrinolytic system (Biggs et al., 1947; Sherry et al., 1959; Truelove, 1951; Fearnley and Lackner, 1955; Billimoria et al., 1959; Sawyer et al., 1960; Ratnoff and Donaldson, 1960; Ogston and Fullerton, 1961; Angelino, 1964; Jang et al., 1964; Ogston and McAndrew, 1964; Cash and Allan, 1967; Menon, 1967), but there have been relatively few studies combining measurements of both these aspects of haemostasis (Guest and Calender, 1960; Bond et al., 1961; Iatridis and Ferguson, 1963; Ikkala et al., 1963; Burt et al., 1964).

From these observations it has become apparent that exercise has an effect on both the coagulation and fibrinolytic systems, and that the degree of change within certain limits, is proportional to the intensity of the stimulus (Ogston, 1961; Rizza, 1961; Angelino, 1964; Cash and Woodfield, 1967; Menon et al., 1967). It is also clear that changes in fibrinolytic tests are more marked than those in the coagulation tests.

(Iatridis and Ferguson, 1963; Burt et al., 1964). Physical exercise causes an acceleration of various in vitro measurements of blood coagulation with the production of a form of hypercoaguability, while tests of fibrinolysis demonstrate an increased potential ability of the blood to lyse fibrin. Some, but not all of the changes in the various coagulation tests can be attributed to the apparent rise of Factor VIII now known to occur after strenuous exercise (Rizza, 1961; Egeberg, 1963), while the alterations in the fibrinolytic system are regarded as reflecting increased levels of circulating plasminogen activator (Sherry, 1959; Iatridis and Ferguson, 1963).

If, as has been suggested (Astrup, 1956), the coagulation and fibrinolytic systems are in a state of balanced equilibrium, then it might be postulated that the two systems are linked by certain components which are common to each. There is evidence to suggest that Factor XII (Hageman Factor) is in some way, related to the endogenous activation of both the coagulation and fibrinolytic systems (Niewiarowski and Prou-Wartelle, 1959; Iatridis, 1961; Iatridis and Ferguson, 1961, 1962, 1964; Holeman and Roberts, 1964; Aznar and Lopez-Borresca, 1965; Aznar and Macarulla, 1965; Kleniewski, 1965; Holeman, McConnell and Johnston, 1966). Experiments in vitro have indicated that surface activation of plasma by a variety of physical agents such as glass or kaolin will accelerate fibrinolysis in euglobulin solutions (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1965; Kleniewski, 1965; Ratnoff, 1966), and this phenomena does not take place if the plasma is deficient in Hageman Factor. Holeman and Roberts (1964) have also shown that Hageman Factor deficient subjects submitted to venous occlusion of the forearm, exhibit a lower fibrinolytic

response than do normal subjects. It has also been noted that Hageman Factor deficient patients have a low resting activity of blood fibrinolytic activity (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1962; Holeman and Roberts, 1964). Also, some purified forms of Hageman Factor have plasminogen activator-like activity (Aznar et al., 1964; Haanen et al, 1964). Surface contact, by way of numerous physical agents can activate the intrinsic pathway of coagulation and this reaction is dependent on the presence of Hageman Factor (Ratnoff, 1961). Thus it appeared on experimental grounds that Hageman Factor might possibly be a common component of both systems.

Further evidence was provided by Iatridis and Ferguson (1963), who in a study of coagulation and fibrinolysis in relation to exercise, found that strenuous activity produced an apparent threefold rise in Factor XII along with marked increase in fibrinolytic activity. In Hageman Factor deficient subjects the fibrinolytic change was very small. It was therefore postulated that there was some dependence of the thrombolytic activity evolved on the amount of activated Hageman Factor available.

The activation of the fibrinolytic system by exercise was studied closely by Cash (1967). Utilizing a standardised moderate exercise procedure on a treadmill, the degree of fibrinolytic activity evoked, as measured by the increase in euglobulin lysis activity, was found to be a reproducible phenomena in normal individuals, and there was a significant intra subject variation in response. A small group of these subjects had an apparently very poor fibrinolytic response to the exercise stress, and it was postulated that in situations of intravascular stress, these individuals might be at risk to conditions such as atherosclerosis, thrombosis and irreversible shock in which a defective fibrinolytic system has been shown

to play some part (Astrup, 1956; Hardaway, 1966). Closer analysis of the publications of other research groups also confirmed the presence in apparently normal populations, of poor fibrinolytic responders (Biggs et al., 1947; Iatridis and Ferguson, 1963; Burt et al., 1964). Iatridis and Ferguson (1963), had attempted to assess the change of Factor XII and fibrinolysis in this group of subjects but without arriving at any firm conclusions.

These previous investigations indicated that exercise studies of the stimulated coagulation and fibrinolytic systems in a group of normal subjects could conceivably lead to an elucidation of the relationship existing between Factor XII and fibrinolysis and as well as accumulating further information on the coagulation changes occurring in the poor fibrinolytic responder. The significant findings, in any individual might not necessarily be the resting level of either the coagulation or fibrinolytic system factors, but the way in which both mechanisms reacted following a standard exercise stimulus. Any detectable latent tendency towards a dis-equilibrium of the coagulation and fibrinolytic response might be of a pathophysiological relevance in those situations that could be associated with an episode of intravascular coagulation. Alternatively, the poor fibrinolytic responder might not be at a disadvantage if it could be shown that the coagulation system was also compensating by a lowered rate of fibrin formation.

In summary, therefore, it was planned to investigate simultaneously, changes in both the coagulation and fibrinolytic systems in a group of apparently healthy subjects, following exercise stress. Reported alterations

in Factor XII activity were to be confirmed and correlations attempted between the coagulation and fibrinolytic test results, and equated with the dynamic equilibrium theory of haemostasis. At the same time, a detailed study of the coagulation system in the poor fibrinolytic responders could be made.

SUBJECTS and EXPERIMENTAL DESIGN.

Prior to commencing experiments on submaximal exercise procedures, studies were required to determine which coagulation tests would yield measurable alterations on exercise. A severe exercise procedure was therefore initially chosen, this stimulus being the most likely to produce marked changes in both the clotting and fibrinolytic systems. It was proposed that coagulation tests demonstrating marked changes on severe exercise were to be used for submaximal exercise studies.

A. Severe exercise study.

Fourteen healthy volunteers (all male) were chosen for the severe exercise study. Eleven of these subjects were aged between 21 and 31 years (mean age 25), the remaining three were known poor fibrinolytic responders, aged between 46 and 49 years (mean age 48). No young poor responders at this time were available.

The group of young subjects were recruited from laboratory staff and other medical personnel; all had satisfactory medical histories. The three middle-aged volunteers were required to give a full medical history, and were submitted to a further medical examination, as outlined in B.

The severe exercise procedure consisted of walking on a treadmill at an elevation of 10° for three minutes at 3.4 m.p.h., followed by three minutes at 3.7 m.p.h. and then one minute at 4.0 m.p.h.; the speed was then increased to 4.2 m.p.h. until the subject removed himself from the treadmill,

and the time required for this varied between one and eight minutes. Each exercise was preceded by an obligatory supine rest period of 30 minutes. Blood samples were taken from an antecubital vein both prior to and immediately following the exercise; in some investigations additional samples were taken over the hour following the exercise. The mean pulse rate increase using this procedure was $192 \pm 13/\text{min.}$, and was similar in both the young and middle-aged subjects.

All experiments were conducted between 9.00 a.m. and 12 noon, after a light breakfast, in a procedure room at $19 - 20^{\circ}\text{C}$. Smoking and excessive exercise before the exercise procedure was discouraged.

B. Moderate exercise study.

From a panel of 50 middle-aged subjects recruited one year previously for fibrinolytic studies, 18 were again approached and asked if a further study exercise could be made. All subjects consented. The selection of subjects was made on the basis of their previously established fibrinolytic response; i.e. low, mid and high fibrinolytic responders were specifically chosen. The number in each category of fibrinolytic response is shown as follows :-

<u>Fibrinolytic response %</u>	<u>No. of subjects</u>
0 - 20	2
21 - 40	7
41 - 60	6
Over 60	3

In this group there were 15 males and 3 females. The age range was

43 to 55 with a mean of 49 years.

These middle-aged volunteers had been previously medically investigated, and the following parameters assessed.

1. Medical history.
2. Medical examination; including clinical examination of heart, peripheral pulses, chest and abdomen.
3. Measurement of resting (supine 15 minutes) blood pressure (by auscultation).
4. Urine analysis for sugar and albumin.
5. Full Blood Count.
6. Resting and post exercise (2 minutes step-up test) electrocardiography.

With this new study, a year later, these parameters were again repeated on an initial visit to the laboratory. In addition, fasting blood samples were obtained for the following tests.

1. Blood glucose.
2. Serum cholesterol.
3. Serum free fatty acids.
4. Serum triglycerides.

These latter tests were included to assess if any subject had latent biochemical evidence of changes commonly associated with cardiovascular disease.

In some individuals changes were seen on the post exercise electrocardiographs in the absence of clinical symptomatology. These E.C.Gs. were referred to a cardiologist for assessment before exercise studies

were commenced, and full details are given in the appendix (page 365).

The standard exercise used was an eight minute walk on a treadmill, moving at a speed of 3.4 m.p.h. at an angle of 5° elevation. Each exercise procedure was preceded by an obligatory rest period, lying down, of 30 minutes, and the same environmental conditions were observed as for the exhaustive exercise procedures. Blood samples were obtained from an ante-cubital vein, with minimal venous occlusion, prior to, and immediately following the exercise. All subjects were studied on more than one occasion with a time interval of at least a week, and often longer, between separate exercise procedures.

RESULTS.

A. Exhaustive Exercise Study.

The results of these experiments are summarised in Tables 4:1, 2, 3 and 4.

(a) Global tests of coagulation (Table 4:1).

There was a significant change in the whole blood clotting time in silicone ($t = 2.553$, $0.05 > p < 0.025$); the recalcified plasma clotting time both with platelet rich plasma ($t = 7.000$, $p < 0.001$) and platelet poor plasma ($t = 5.660$, $p < 0.001$); and the kaolin-cephalin clotting time ($t = 4.585$, $p < 0.001$). No significant change was seen in the prothrombin time or thrombin time.

The percentage change in the whole blood clotting time (mean 9%), and the kaolin-cephalin clotting time (mean 8%) could be explained by the alteration in haematocrit occurring post exercise (mean change 8%). There was a significant correlation between the K.C.C.T. and haematocrit ($r = 0.79$, $0.02 > p > 0.01$). No significant correlations were found between the percentage change between each type of clotting test. The changes in the coagulation tests of the three poor fibrinolytic responders (subjects 5, 6 and 7) were of a similar order to that of the other subjects examined.

(b) Factor Assays (Table 4:2).

Significant increase occurred in the levels of fibrinogen ($t = 3.60$, $0.01 > p > 0.005$), Factor VIII ($p < 0.001$), and H.P.F. ($t = 4.463$, $0.01 > p > 0.02$).

TABLE 4:1.

THE EFFECT OF A SEVERE EXERCISE PROCEDURE ON THE GLOBAL TESTS OF COAGULATION,
AND ON HEPARIN PRECIPITABLE FRACTION, IN FIFTEEN SUBJECTS.

TEST	Whole Blood Clotting Time (Minutes)				Recalcified Plasma Clotting Time (Seconds)				Kaolin-cephalin Clotting Time (Seconds)		Prothrombin Time (Seconds)		Thrombin Time (Second)		Heparin Precipitable Fraction (H.P.F.) mgms/100 ml.	
	Glass		Silicone		Platelet Rich		Platelet Poor									
Subject	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
1	6.6	6.0	20.6	16.0	120	104	149	112	36.2	34.1	15.1	15.1	12.0	12.0	-	-
2	7.5	6.0	17.1	15.6	117	84	174	107	37.3	35.4	14.9	15.1	13.1	12.0	35.2	44.9
3	8.5	8.1	21.4	20.0	127	105	281	134	42.5	40.0	13.8	14.2	12.5	12.6	-	-
4	9.2	8.0	16.0	17.1	100	98	201	155	34.1	30.8	16.1	15.4	13.2	13.9	-	-
5*	9.0	10.0	22.4	20.1	92	56	190	120	38.2	35.3	-	-	-	-	-	-
6*	6.2	5.9	16.1	14.2	125	111	171	156	35.1	33.2	13.8	13.8	14.2	14.1	-	-
7*	11.0	10.7	21.9	21.0	104	93	180	132	40.2	37.1	14.0	14.0	15.0	15.8	-	-
8	-	-	-	-	118	99	196	173	37.6	35.0	15.0	15.1	12.0	12.8	38.7	67.9
9	-	-	-	-	110	98	231	181	39.2	36.3	14.9	15.0	13.2	13.3	29.0	45.0
10	-	-	-	-	140	119	292	192	40.6	36.1	14.0	12.8	-	-	34.0	59.0
11	-	-	-	-	-	-	-	-	39.0	38.1	-	-	12.3	12.4	34.0	54.0
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	44.3	97.4
14	-	-	-	-	125	108	184	132	35.5	34.5	14.0	14.0	-	-	-	-
15	-	-	-	-	135	112	151	107	42.5	32.0	-	-	-	-	35.0	41.4
Mean ± S.D.	8.3 ±1.7	7.8 ±2.0	19.4 ±2.8	17.7 ±2.6	118 ±14	99 ±16	200 ±46	142 ±29	38.3 ±2.7	35.2 ±2.5	14.6 ±0.8	14.4 ±0.8	13.0 ±1.0	13.2 ±1.2	35.7 ±4.7	58.5 ±19.5
t Test	t = 1.5604 N.S.		t = 2.5525 0.05 > p > 0.025		t = 7.000 p < 0.001		t = 5.6600 p < 0.001		t = 4.5854 p < 0.001		t = 0.730 N.S.		t = 0.7885 N.S.		t = 4.4627 0.01 > p > 0.002	

* 'Poor Fibrinolytic Responders'
N.S. = Not Significant

B = Before Exercise
A = After Exercise

TABLE 4:2.

THE EFFECTS OF A SEVERE EXERCISE PROCEDURE ON THE ASSAYED LEVEL OF COAGULATION FACTORS, IN FIFTEEN SUBJECTS

Coagulation Factor	I		II	V	VII	VIII	IX	X	XII	XIII	"Contact Factor" Activity
Subject	Before Exercise	After Exercise	Figures are Expressed as a percentage of the pre exercise value (%).								
	mgm/100 ml.										
1	-	-	-	-	-	131	-	-	105	-	-
2	-	-	110	115	-	-	-	109	98	-	-
3	-	-	105	121	-	-	-	-	99	-	-
4	-	-	113	107	-	-	-	-	143	-	-
5*	263	284	-	105	-	142	-	-	79	-	-
6*	221	226	-	-	-	196	-	-	125	-	-
7*	373	437	-	-	-	170	-	-	89	-	-
8	204	221	89	-	-	181	106	100	-	-	-
9	162	174	-	98	-	135	115	96	-	-	-
10	155	213	-	-	106	214	92	103	-	-	-
11	154	169	-	-	97	252	103	110	-	-	-
12	-	-	107	110	-	-	-	-	98	-	-
13	169	187	-	-	101	160	97	82	-	115	110
14	-	-	100	-	89	181	90	120	-	95	120
15	152	168	-	112	109	303	-	-	-	116	103
Mean % S.D.	205 ±73	231 ±85	104 ± 8	109 ± 7	100 ± 7	187 ±52	100 ± 9	102 ±12	- -	109 -	111 -
Significance	t = 3.600 0.01 > p > 0.005		t = 1.142 N.S.	t = 3.4778 0.02 > p > 0.01	t = 0.1137 N.S.	t = 5.5535 p < 0.001	t = 0.1303 N.S.	t = 0.6267 N.S.	t = 0.6244 N.S.	t = 1.2671 N.S.	t = 2.2296 N.S.

* 'Fibrinolytic Poor Responders.'

N.S. = Not Significant.

The percentage change in fibrinogen (8%) was similar to the percentage increase of haematocrit (8%). Factor VIII was increased post exercise to a mean of 130% but the percentage rise was not significantly correlated with the percentage increase in fibrinogen or other global clotting tests. Subjects 5, 6 and 7 (poor fibrinolytic responders) gave normal increases in the levels of Factor VIII. The percentage change in H.P.F. was 61% of the baseline value; this increase did not show a significant correlation with the percentage increase in fibrinogen.

Small changes occurred in the levels of other clotting factors but none reached significant levels and were of no greater extent than would be expected from the alteration in haematocrit. Of particular note, was the absence of a significant change in Factor XII levels.

(c) Fibrinolytic Tests. (Table 4:3).

The changes in the fibrinolytic system are listed in Table 4:3. There was a significant decrease in the euglobulin lysis time ($t = 3.0136$, $0.01 > p > 0.005$) and the mean percentage response (including the three poor fibrinolytic responders 5, 6 and 7) was $75 \pm 15\%$. These changes were reflected in the fibrin plate assays (Table 4:3).

Despite the marked increase in plasminogen activator levels, as evidenced by the greatly shortened euglobulin lysis time, there was no significant difference ($t = 1.3707$) post exercise, in the values of fibrin/fibrinogen degradation products, in 7 subjects.

The mean percentage change in fibrinolysis, as assessed by the estimations of the euglobulin lysis time was approximately three times the percentage change in coagulation as indicated by recalcification time of

TABLE 4:3.

THE EFFECT OF SEVERE EXERCISE PROCEDURE ON FIBRINOLYSIS IN FIFTEEN SUBJECTS

Subject Number	Euglobulin Lysis Time (Minutes)		% Response	Fibrin Plate Assay Method (mm ² of lysis)						Fibrin Degradation Products (μgms/ml.)	
				Human Fibrinogen		Bovine Fibrinogen		Plasma Drops Human Fibrinogen			
	B*	A		B	A	B	A	B	A	B	A
1	120	22	88	-	-	-	-	-	-	-	-
2	141	16	89	400	900	105	528	100	625	-	-
3	104	20	80	-	-	-	-	-	-	-	-
4	231	43	81	-	-	-	-	-	-	-	-
5**	765	413	46	100	240	100	144	49	64	-	-
6**	525	300	52	225	380	100	100	0	0	-	-
7**	730	340	53	289	324	64	81	0	0	-	-
8	135	40	70	455	700	121	225	138	360	3.6	14.3
9	106	41	61	473	675	100	181	121	225	7.2	5.4
10	99	23	77	361	702	132	398	100	400	9.0	12.8
11	63	15	76	546	1422	121	599	100	729	9.3	12.3
12	321	59	82	-	-	-	-	-	-	-	-
13	592	71	88	275	710	-	-	56	182	5.4	3.6
14	397	26	93	494	1177	-	-	100	700	10.8	12.6
15	338	28	92	335	1165	-	-	-	-	5.4	5.4
Mean S.D.	311 ±240	97 ±134	75 ±15	359 -	7638 -	105 -	282 -	76 -	329 -	7.2 -	9.5 -
Significance	t = 5.3467 p < 0.001			t = 4.6488 p < 0.001		t = 2.8367 0.05 > p > 0.025		t = 3.3714 0.01 > p > 0.005		t = 1.3707 Not significant	

* Abbreviations B = Before Exercise
A = After Exercise

** Fibrinolytic 'Poor Responders'

TABLE 4:4.

THE EFFECT OF SEVERE EXERCISE ON SPECIFIC COAGULATION FACTORS

	I	II	V	VII	VIII	IX	X	XI	XII	XIII
Rizza (1961) 15 subjects	No Change	No Change	No Change	-	Average increase 219% (Two stage assay)	No Change (Two stage method)	-	-	-	-
Ratnoff (1961) 12 subjects	No Change	-	-	-	-	-	-	-	-	-
Ikkala (1963) 10 subjects	No Change	-	Significant increase 100-200%	No Change	120-540% (One stage assay) Average increase	No Change (One stage method)	No Change	"Contact Factor Activity" 240-1000%		-
Egeberg (1963) 5 subjects	No Change	No Change	No Change	No Change	2-3x increase (One stage assay)	No Change (One stage method)	-	No Change	Slight Increase	-
Iatridis and Ferguson (1963) 59 subjects	No Change	No Change	No Change	No Change	188% (Two stage assay) Average increase	-	No Change	-	Average Increase 318%	-
Veltkamp (1967) 9 subjects	-	-	-	-	158% Average increase (One stage assay)	No Change (One stage method)	-	-	No Change	-
Woodfield (1968) 15 subjects	No Change	No Change	No Change	No Change	187% Average Increase (One stage assay)	No Change (One stage method)	No Change	No Change	No Change	No Change

plasma. This observation is similar to that of Burt et al. (1964).

The mean percentage decrease of the euglobulin lysis time in subjects 5, 6 and 7 was 50%, as compared with a mean of 81% in the other subjects.

There were no significant positive correlations between the percentage decrease in euglobulin lysis times and the percentage change in any of the clotting tests.

B. Moderate Exercise Study.

The results of the severe exercise study indicate that submaximal studies of coagulation would be of limited value as only a few coagulation tests would yield significant changes. The moderate exercise experiments were therefore orientated towards a further assessment of fibrinolysis with a few selected coagulation tests included.

(a) Fibrinolytic Tests.

The results of the fibrinolytic study in the eighteen selected middle-aged subjects are shown in Table 4:5. The mean pre exercise baseline euglobulin lysis time was 251 ± 222 minutes, and there was no statistical difference between this value and that recorded in the study one year earlier ($t = 0.5594$, $p = \text{N.S.}$). A good correlation was also found between both the individual resting euglobulin lysis times, the percentage responses, and the previous studies of these parameters ($r = 0.84$, $p < 0.001$; $r = 0.86$, $p < 0.001$, respectively). The correlation between the fibrinolytic responses in the two studies is illustrated in Fig. 4:1 and tabulated in Table 4:6.

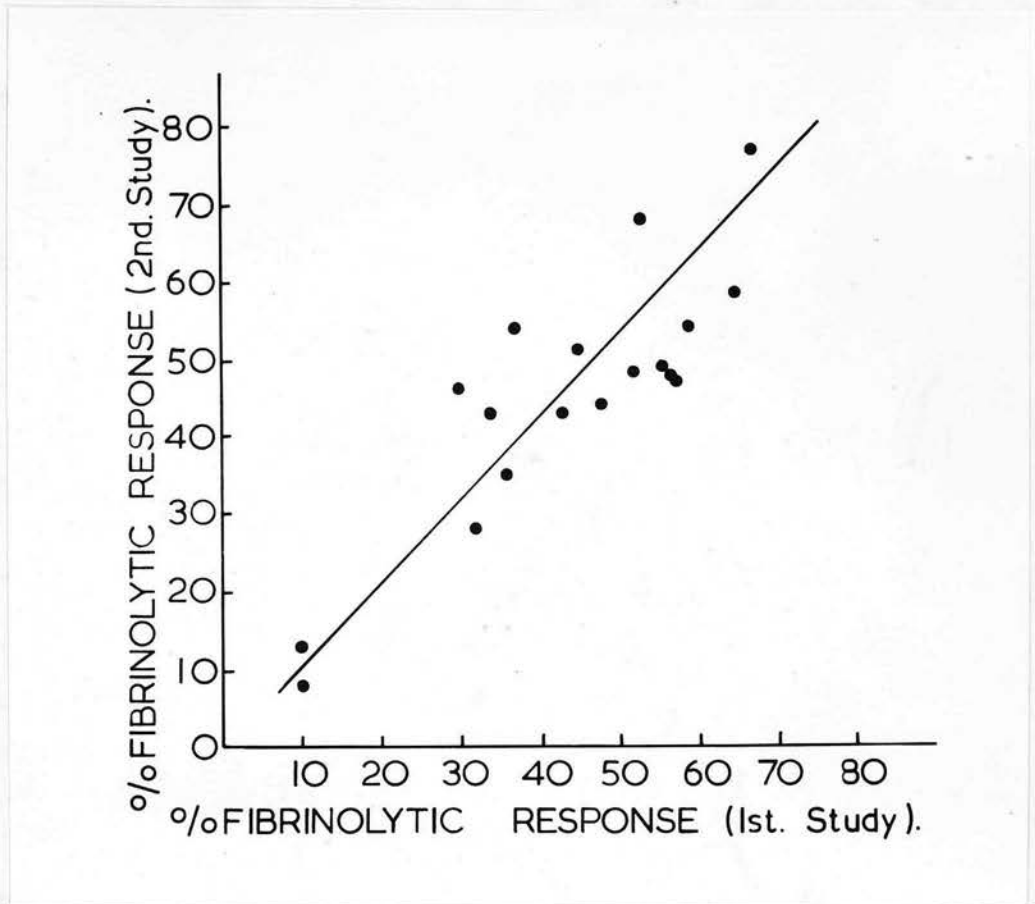


Fig. 4:1. Correlation between the percentage fibrinolytic responses in two separate studies performed one year apart.

TABLE 4:5.

THE RESULTS OF THE EUGLOBULIN LYSIS TIME ESTIMATION
BEFORE AND AFTER EXERCISE IN EIGHTEEN MIDDLE-AGED SUBJECTS

Subject	Euglobulin Lysis Time		% Response	Mean % Response	Subject	Euglobulin Lysis Time		% Response	Mean % Response
	Before	After				Before	After		
M 1 (M 9)	172 307 343	107 164 132	38 47 62	49	M 10 (M 23)	330 628	320 546	3 13	8
M 2 (M 12)	227 264	117 121	48 54	51	M 11 (M 14)	252 362	154 189	39 48	44
M 3 (M 1)	620 542	530 489	15 10	13	M 12 (M 3)	180 59	89 34	51 42	47
M 4 (M 5)	186 330 257	62 211 153	67 36 40	48	M 13 (M 7)	347 188	186 104	46 45	46
M 5 (M 27)	152 121	82 73	46 40	43	M 14 (M 10)	265 257	82 81	69 68	68
M 6 (M 20)	150 231	95 118	37 49	43	M 15 (M 11)	186 252	101 99	46 61	54
M 7 (M 21)	370 434	190 345	49 21	35	F 1 (F 9)	182 299	131 215	28 28	28
M 8 (M 6)	334 287	157 166	53 42	48	F 2 (F 25)	223 144	38 41	83 72	77
M 9 (M 22)	265 490	96 275	64 44	54	F 3 (F 16)	429 262	151 131	65 50	58
					t test t = 4.0875, p < 0.001				

Figures in parenthesis refer to subject number of previous study (Cash, 1967)

TABLE 4:6

COMPARISON OF THE RESTING EUGLOBULIN LYSIS TIMES BEFORE AND AFTER MODERATE EXERCISE, AND THE PERCENTAGE RESPONSES, IN EIGHTEEN MIDDLE-AGED SUBJECTS, IN TWO SEPARATE STUDIES.

Subject	Mean resting euglobulin lysis time (minutes)		Mean percentage response	
	1st. study	2nd study	1st study	2nd. study
M 1	125	274	56	49
M 2	79	246	45	51
M 3*	865	581	10	13
M 4	79	258	57	48
M 5	140	137	34	43
M 6	109	191	43	43
M 7	596	402	36	35
M 8	218	311	52	48
M 9	202	378	59	54
M10*	593	479	10	8
M11	429	307	48	44
M12	173	120	58	47
M13	114	268	30	46
M14	245	261	53	68
M15	107	219	37	54
F 1	104	240	32	28
F 2	125	184	68	77
F 3	206	346	65	58
Mean + S.D.	251 ± 222	289 ± 116	44.1 ± 17	45.2 ± 17
"t"test	t = 1.1743 (Not significant)		t = 0.5594 (Not significant)	
Correlation Coefficient	r = 0.84 (p < .001)		r = 0.86 (p < .001)	

* Poor fibrinolytic responder

The results of the F.D.P. assays are summarized in Table 4:7.

There is no significant increase post exercise ($t = -1.1941$, $0.3 > p > 0.2$).

Some of the assays were repeated on several occasions, by separate technicians, and the findings were confirmed.

(b) Coagulation Tests .

There were no significant changes in the pre and post exercise plasmas using the kaolin-cephalin clotting time, the recalcified plasma clotting time of platelet rich plasma, Factor V, VIII or XII levels.

A test showing a significant change was the recalcified plasma clotting time on platelet poor plasma (Table 4:8). Experiments using a modified styphen time test suggested that the shortening of clotting time observed could be due to a phospholipid-like activity (similar to platelet Factor III activity) appearing in the plasma post exercise. There was no significant correlation in the percentage changes in this clotting time compared with the fibrinolytic response ($t = 0.3267$).

There was again a significant increase in the level of heparin precipitable fraction ($t = 2.2544$, $0.05 > p > 0.025$) (Table 4:9).

Reproducibility of baseline values and response by individuals was poor and there was no correlation with the fibrinolytic response.

(c) Biochemical Changes.

These are listed in Table 4:10. Most results are within the normal ranges for fasting serum samples. Two poor (M3 and M10), and two high responders (M12 and F3) had levels of serum triglycerides above the normal limits but in each case serum cholesterol were normal as were the

FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS (F.D.P.s) ASSAYED PRIOR TO AND IMMEDIATELY
FOLLOWING A MODERATE EXERCISE PROCEDURE

Subject	Before	After	Subject	Before	After	Subject	Before	After
μ gms./ml.			μ gms./ml.			μ gms./ml.		
M 1	13.7 4.5	14.5 5.4	M 8	7.2 10.8	10.8 5.4	M15	8.0 4.5	8.0 4.5
M 2	6.5 14.4	8.4 10.8	M 9	8.6 13.3	17.3 20.3	F 1	5.8	17.3
M 3*	17.3 7.2	17.3 6.3	M10*	12.0 7.7	18.0 8.8	F 2	5.4 4.0	10.8 8.1
M 4	9.0 9.3 4.5	9.0 9.7 5.4	M11	7.2	3.6	F 3	4.2	5.2
M 5	11.5 3.6	11.5 5.4	M12	9.0 4.5	6.0 4.5	Mean S.D.	7.8 +3.8	9.0 +4.8
M 6	4.3 8.6	11.5 8.6	M13	5.4 3.6	5.4 3.6	"t" test	$t = 1.194$ $0.3 < p < 0.2$	
M 7	6.3 14.4	7.2 7.2	M14	1.4 3.6	1.8 5.4			

* Poor fibrinolytic responder

TABLE 4:8.

THE EFFECT OF A STANDARDISED MODERATE EXERCISE PROCEDURE ON THE RECALCIFICATION TIME (SECONDS) OF PLATELET POOR PLASMA IN FIFTEEN SUBJECTS (30 OBSERVATIONS)

Subject	Before	After	% Response	Subject	Before	After	% Response
M 1	199 199	182 172	9 14	M 9	218 260	172 219	21 16
M 2	216 162	185 138	14 15	M10*	170 190	152 171	11 10
M 3*	198 186	172 171	13 8	M11	171 182	149 149	13 18
M 4	176 193	174 173	1 10	M12	237 215	219 166	8 23
M 5	201 180	172 150	14 17	M13	195 152	179 129	8 15
M 6	212 190	204 159	4 16	F 1	183 197	167 163	9 17
M 7	223 174	200 151	10 13	F 2	214 198	170 139	21 30
M 8	155 166	148 142	5 14	Mean	194	168	13
				S.D.	± 24	± 22	± 6
				"t"test	t = 4.3168 p < .001		

* Poor fibrinolytic responder

TABLE 4:9.

THE CHANGES IN HEPARIN PRECIPITABLE FRACTION (mgms./100 mls.) AFTER A MODERATE EXERCISE PROCEDURE IN SEVENTEEN SUBJECTS (27 OBSERVATIONS)

Subject	Before	After	% Change	Subject	Before	After	% Change
M 1	71.7 72.0	84.1 75.0	17 4	M10*	30.0 46.0	40.0 45.0	33 0
M 2	66.5 46.0	86.4 50.0	23 9	M11	51.0	57.0	12
M 3*	65.0 36.0	72.0 48.0	11 33	M12	41.0	52.0	27
M 4	40.0 51.0	50.0 54.0	25 6	M13	26.5	32.2	21
M 5	43.0	50.0	16	M14	47.0 32.0	48.0 47.0	2 47
M 6	55.0 31.6	60.0 37.0	9 17	M15	44.2 33.0	47.6 36.4	8 10
M 7	39.0	56.0	44	F 1	35.0 40.4	37.0 46.4	6 15
M 8	28.0	48.4	72	F 3	36.0 32.0	46.0 47.0	28 47
M 9	55.0	63.0	15	Mean	44.2	52.4	20.6
				S.D.	± 13.1	± 13.6	± 16.8
				"t"test	$t = 2.2544$ $0.05 < p < 0.025$		

* Poor fibrinolytic responder

serum F.F.A. Two subjects (M4 and M6) had levels of serum F.F.A. above the normal range but no other biochemical abnormality. One fibrinolytic good responder had an elevated cholesterol value (F2), with an elevated F.F.A. The significance of these isolated changes is not known.

Subject
Number
M 1
M 2
M 3*
M 4
M 5
M 6
M 7
M 8
M 9
M 10*
M 11
M 12
M 13
M 14
M 15
F 1
F 2
F 3

TABLE 4:10.

RESULTS OF BIOCHEMICAL TESTS ON EIGHTEEN MIDDLE-AGED SUBJECTS (FASTING)

Subject Number	Plasminogen Activator Level (Units)	Serum Cholesterol mgms. % (Normal range 150-290.)	Serum Triglycerides in mM/l (Normal range 40-120 mgms/100 ml.)	Serum Free Fatty Acids mEq/l (Normal range 500-700 mEq/l)	Blood Glucose Upper limit of Normal 120 mgms. %
M 1	7.9	251	110	754	60
M 2	6.8	193	117	696	60
M 3*	1.6	220	168	529	102
M 4	11.9	185	83	1019	75
M 5	8.6	231	103	745	78
M 6	11.7	208	110	1009	90
M 7	4.1	195	125	754	84
M 8	3.1	226	129	619	94
M 9	4.0	261	106	862	74
M 10*	6.6	215	250	-	68
M 11	4.5	274	118	552	92
M 12	7.6	241	191	381	87
M 13	11.7	251	72	750	90
M 14	4.0	230	100	361	105
M 15	14.3	221	117	532	87
F 1	10.7	352	75	664	84
F 2	4.7	200	93	931	100
F 3	3.9	225	146	767	73

* 'Fibrinolytic Poor Responders.'

DISCUSSION.

Severe, acute exercise procedures will produce changes in both global tests of coagulation, some specific factor assays, and in fibrinolysis. The results of this small study have confirmed that these changes take place but also indicate that inter relationships of coagulation and fibrinolysis will be difficult to clarify using an exercise stimulus, particularly if it is to be controlled and submaximal.

Global tests of coagulation would appear superficially attractive to assess the overall changes in blood coagulability. One of the first such tests to be described was that of the whole blood coagulation time (Lee and White, 1913; Mills and Necheles, 1928), and with its varying modifications (Hartman, 1927; Biggs and Macfarlane, 1962), it has been used extensively. This test, although simple in principle, is profoundly affected by small variations in technique, and even with careful standardisation it can be insensitive to wide variations of blood coagulability. Small alterations in clotting times are therefore difficult to interpret. Nevertheless, using it as an estimation of post-severe exercise coagulability a significant shortening of the clotting time in silicone coated tubes was detected. This was, however, compensated for by an equivalent increase in haematocrit; the change therefore, is unlikely to be significant. This result is similar to that of Schneider and Zangara (1951); Rizza (1961) and Keeney and Laramie (1962), but is not in agreement with Iatridis and Ferguson (1963); and Finkel and Cumming (1965). In the latter study, no allowance

was made for haemoconcentration and in the former, the sensitivity was higher, for reasons not clearly stated, than is usual with the Lee and White whole blood clotting time. Besides variations in technique it is possible that environmental changes can affect the whole blood clotting time test, as Finkel and Cumming (1965) demonstrated that severe exercise at $+25^{\circ}\text{C}$ produced a slight change in clotting time, but exercise at -20°C did not.

A significant difference, pre and post exercise, in the recalcification time of both platelet rich and platelet poor plasmas was demonstrated after severe exercise and this finding is similar to that of Burt (1964); Ikkala, (1963); and Iatridis and Ferguson (1963). The mean percentage decrease of clotting time as reported by Burt was 24%; in our study it was 28%. Ikkala (1963), tested platelet poor plasma and a statistically significant decrease in recalcification time persisted for up to 60 minutes post exercise. In both these global tests of coagulation, there was no correlation between the percentage change in the recalcification time and the percentage rise of Factor VIII, and the correlation between the two methods of recalcification time technique was also poor ($r = 0.4719$, $p = \text{N.S.}$) It is considered that the inherent errors of these types of tests make them unsuitable for evaluating small changes within the normal range of values.

Using the remaining global test of coagulation, the kaolin-cephalin clotting time, a small post severe exercise increase in coagulability could be demonstrated. The change was similar to that of the haematocrit (8%) and there was a significant correlation between the two tests ($r = 0.7928$, $0.02 > p > 0.01$). There was no correlation in the percentage change between

this test and the other clotting tests. These results are similar to those of Iatridis and Ferguson (1963); Egeberg (1963); and Finkel and Cumming (1965), who all reported a shortening of the partial thromboplastin time, a test in which contact activation is only partly controlled. It might be considered that this test would be sensitive to increased levels of Factor VIII; but the correlation was low ($r = 0.5927$, $0.1 > p > 0.05$), and overshadowed by the haematocrit change.

Coagulation tests based upon measurements of changes in the extrinsic system of coagulation, e.g. the prothrombin time test, did not show a post exercise alteration (Rizza, 1961; Iatridis and Ferguson, 1963; Ikkala et al., 1963). The results obtained are similar.

The assay of specific coagulation factors was of interest. In most clotting factors, there was no significant alteration. Factor VIII, however, was unequivocally raised after severe exercise, as assayed by a one-stage assay system. This is in agreement with other recent work (Rizza, 1961; Ikkala, 1963; Veltkamp, 1968), and may be due to potentiation of Factor VIII activity in the blood stream (Ingram, 1965), or to splenic release (Webster et al., 1967) secondary to altered circulatory haemodynamics. Moderate exercise did not significantly raise the level of Factor VIII and from other studies it is apparent that a severe exercise procedure is required (Rizza, 1961; Veltkamp, 1968). There was no significant correlation between the percentage change in Factor VIII levels and the alteration in fibrinogen or other clotting tests.

An interesting finding was the absence of a significant increase in Factor XII levels post exercise. This is not in accordance with Iatridis

and Ferguson (1963), who first detected a change. The explanation may lie in differences of methodology. The two stage assay system of Iatridis and Ferguson (1962a), is a modification of the Hicks and Pitney (1957) method for the assay of Factor VIII. Describing their method, Iatridis and Ferguson express reservations about the very wide range of normal values found and suggest that optimal kaolin activation at 4°C might improve the reproducibility of the assay. No further reports describing their assay technique have appeared; and attempts to obtain reproducibility of the assay in this laboratory were not successful, in spite of considerable effort. Criticisms of the test include the use of non-siliconized apparatus, and the consequent poor control of surface activation phenomena. The test is also sensitive to suboptimal levels of phospholipid in the incubation mixture as well as to relative decreases of Factor V and VIII, these latter being supplied by the Factor XII deficient substrate plasma. Another source of variation is the apparent sensitivity of the assay system to A.T.Pases, enzymes derived from platelets (Ferguson et al., 1967). The rise, post exercise, of Factor XII as first reported, must be questioned as the technique is unusual and dissimilar to methods more widely used for the assay of Factor XII. Egebers (1963), found small rises of Factor XII post exercise but only after repeated exercising; in the assay he describes, surface activation is poorly controlled and again the results must be interpreted with caution. Ikkala (1963), found a post exercise increase in "Contact Product", i.e. combined Factor XI and XII activity as prepared by the method of Waaler (1959). Using Nossel's method (1964), which is similar to that of Waaler, this increase of contact product could not be confirmed in several experiments in this laboratory (Table 4:2). It is also of relevance to note

that Factor XI, the other coagulation contact factor, does not alter in concentration post exercise (Egeberg, 1963).

These other studies, therefore, though not strictly comparable, are all open to question as to specificity of the changes described; more recent work by Veltkamp (1968) has shown that Factor XII, as assessed by assays based upon the method of Hardisty and Macpherson (1962) is not increased post exercise. However, it is still possible that these other assay techniques may be measuring facets of coagulation that are as yet incompletely understood or may be measuring Factor XII in an activated state. Thus the study of Factor XII in this type of experimental situation did not yield positive information on the postulated relationships between coagulation and fibrinolysis.

It is perhaps relevant to note, at this juncture, that the envisaged relationship existing between Factor XII and the activation of fibrinolysis, relies substantially on in vitro experiments. Niewiarowski and Prou-Wartelle (1959) and Iatridis and Ferguson (1960, 1962b) incubated normal plasma euglobulin with kaolin and observed an increase in lytic activity. This effect did not occur in plasma from patients with Factor XII deficiency. Aznar (1965) incubated a preparation of "contact factor" with streptokinase and found lytic activity was evolved but it is probable that plasminogen contamination could have easily occurred. As mentioned above, the in vivo post exercise rise of Factor XII must be reconsidered seriously and experiments such as those designed by Holemans et al., (1966) using venous occlusion as an in vivo activator of fibrinolysis, on both normal and a very small number of Factor XII deficient patients, were not conclusive as to the part played by Factor XII. Ratnoff (1966) confirmed

the observations of Niewiarowski and Iatridis, but also noted that Factor XI deficient plasma, when completely depleted of Factor XI with celite (but containing normal quantities of Factor XII and plasminogen), did not generate fibrinolytic activity on incubation with kaolin. Also, neither human nor bovine preparations are fibrinolytic in vitro (Ratnoff and Davie, 1962; Schoenmakers et al., 1963). Even more disturbing to the postulated relationship, is the observation that when ellagic acid, a potent in vitro and in vivo specific activator of Factor XII (Botti and Ratnoff, 1964; Girolami et al., 1966) is injected into animals or humans, no enhancement of activator activity occurs, even though a marked hypercoagulable state is produced. It is apparent that evidence for Factor XII either in the "non activated" or "activated" form being able to initiate the activation of plasminogen is not yet strong, and much further work is required to reconcile the contradictory in vitro and in vivo experimental results. In particular the in vitro findings of Iatridis and Ferguson (1962), who are the main protagonists of the concept of Factor XII being involved in the activation of the fibrinolytic system, need to be equated with the negative in vivo results of Ratnoff et al. (1966), who are highly experienced in this field. It is still quite possible, however, that Factor XII may be indirectly linked in a manner yet to be elucidated, with fibrinolysis, but considerable clarification will be needed.

Heparin precipitable fraction was significantly increased both after severe and moderate exercise procedures, and this has not previously been reported. The increase of H.P.F. was not correlated with the increase in fibrinogen, or with any of the other clotting tests. It is possible that this test is revealing a physiological degree of increased coagulability, for,

if H.P.F., at least in part, represents a form of cryoprofibrin in which fibrinopeptide A has been removed by the action of thrombin (Shainoff and Page, 1962), increased levels post exercise could be interpreted as being the result of in vivo thrombin activity, possibly associated with the degree of hypercoagulability.

The interpretation of these small changes occurring in both global and specific factor coagulation tests is problematical. It was not possible to achieve a consistently reproducible change in individuals tested at different times in spite of vigorous technical control of clotting tests, and the alterations were, in most cases, small. Investigation of the fibrinolytic "poor responders" gave essentially similar results to other subjects in this admittedly small study. This type of investigation may require techniques of finer discrimination, sensitivity and specificity before definitive conclusions can be achieved. The assay of in vivo peptides released when fibrinogen is proteolytically digested by thrombin, would provide one possible avenue of relevant research.

Subsequent to both moderate and severe exercise experiments the level of euglobulin lysis activity increases sharply. This increase is less marked in the poor fibrinolytic responders and the changes are also reflected in the fibrin plate assays. Despite these changes, there was no significant increase in the assayed level of fibrin/fibrinogen degradation products post exercise. However, in five of the severe exercise experiments, a small increase was noted; in the moderate exercise studies there was a mean small increase post exercise which did not, however, reach significant levels. These results are at variance to preliminary studies reported by Das et al.

(1967). The explanation of this is not yet clear; minor alterations in technique have undoubtedly occurred since these earlier experiments, which perhaps have affected the discrimination of the assay in detecting small F.D.P. changes within the physiological range. However, using the present F.D.P. method, it is clear that following both exercise and adrenalin infusions (Section 4:2) it was not possible to demonstrate a significant alteration in the circulating levels of F.D.Ps.

If F.D.Ps. do not increase after exercise it could be considered that this was evidence mitigating against the theory of a dynamic balance between coagulation and fibrinolysis. However, it is conceivable that post exercise proteolysis of fibrin may have occurred, although not as far as the plasmin resistant moieties which are measured in the F.D.P. assay. The evidence for this is meagre, although Fisher et al. (1967) have demonstrated high molecular weight thrombin clottable proteolytic fragments of fibrinogen in the sera of patients in whom pathological lysis was occurring, before there was other biochemical evidence of any change. It is not known if similar products occur in stimulated physiological situations. On the other hand, if a 5 μ gm. increase of F.D.Ps. does occur post exercise this would represent in a 70 kilogramme subject a total body lysis of 30 mg. of fibrinogen; a not inconsiderable amount; such calculations, however, are based on the unlikely assumption that the immuno-reactivities of F.D.P. and fibrinogen are equal, and may not be valid (Merskey et al., 1966; Israels et al., 1968).

These conflicting results will only be resolved when more sensitive techniques are available for measuring fibrin proteolysis products. The present assay is not suitable, without further refinement, for detecting

small physiological alterations in circulating levels of F.D.P., and techniques such as radioimmuno-assay may prove valuable research tools.

The significance of an increase in plasminogen activator to an exercise stimulus, as based on these F.D.P. results, must be questioned. The lysis of fibrin may not necessarily be increased post exercise, although the potential for lysis could be. It is suggested that the measurement of in vivo fibrinolysis is perhaps better assessed by the measurement of F.D.P. and it is possible that "fibrinolytic activity" is not synonymous with changes in plasminogen activator level, as has previously been assumed.

It is apparent that the fibrinolytic response of an individual is a verifiable phenomena, and that over the time period of one year, no significant changes occurred in the individual responses. However, we are only a little further forward in our understanding of the significance of the fibrinolytic response to exercise. The coagulation investigations have not substantially clarified the phenomena, and if coagulation and fibrinolysis are linked, the relationship may be complex, requiring techniques as yet not available, such as assays which are of greater specificity and sophistication. Also, although it is possible that the post exercise rise of plasminogen activator is a response to a low grade episode of intravascular coagulation, these studies do not yield definite evidence in either direction. The biochemical investigations of the poor fibrinolytic responders also do not add significantly to the pathophysiological interpretation, although further follow-up studies will be of value. It was of interest to note that although one poor responder (M3) developed definite ischaemic changes on the electrocardiograph, similar changes were also detected in a good responder (F2), and in both these subjects, the changes had not been present

one year previously. Also, one of the original good responders (68%) of the first study died after a posterior myocardial infarct. Necropsy revealed wide-spread coronary atheroma, and microscopy of the right coronary artery (Fig.4:2) showed a thrombus which had probably occurred secondary to haemorrhage into an atheromatous plaque.

It is thus perhaps premature to attach too much importance to the conception of a fibrinolytic response being of importance in pathophysiological events. The phenomena is probably a measure of both the release and removal of circulating plasminogen activator. In vivo thrombolysis is not necessarily dependent on levels of plasminogen activator, for the ability of the activator to dissociate from its anti-activator, the rapidity with which it can be adsorbed into a clot, the intra clot concentration and reactivity of plasminogen, antiplasmins and anti-activators are almost certainly important factors contributing to in vivo clot dissolution. It is quite possible also that intravascular lysis can proceed normally with extremely low levels of plasminogen activator and this is suggested by the normally low levels of plasminogen activator in pregnancy and the lack of untoward clinical complications when E.A.C.A., a plasminogen activator inhibitor, is given to normal subjects. However, although the variations in fibrinolytic response may be only a reflection of the frequency distribution pattern expected in a normal population, with the "poor responders" at one end of the curve, modification of the response by factors such as diabetes (Cash and McGill, 1968), mental stress (Cash and Allan, 1967), and pregnancy (Woodfield et al., 1968) does suggest that the phenomena is of either physiological or pathological importance and warrants further intensive



Fig. 4:2. Photomicrograph of a section of the right coronary artery showing haemorrhage into an atheromatous plaque and thrombosis.

study of other influencing factors. If this can be achieved as a prospective study and aligned with additional investigations of other relevant parameters of vascular integrity, eventual clarification of these problems may be possible.

SUMMARY.

The coagulation and fibrinolytic changes following both a severe and a standardised exercise procedure have been studied. The phenomena of the fibrinolytic response was verified, and poor fibrinolytic responders were again identified. There was no evidence that the coagulation system in these subjects differed from other individuals. There was no apparent correlation in the subjects studied between the coagulation and fibrinolytic tests, and the rise of plasminogen activator was not accompanied by an increase in the concentration of Factor XII. Lysis of fibrin, as assessed by the measurement of its degradation products, was not increased significantly after moderate or severe exercise. It is suggested that exercise studies of coagulation and fibrinolysis are hampered by the imprecise technical tests and further evaluation of the postulated dynamic equilibrium of coagulation and fibrinolysis using this approach may need to await improved methodology.

CHAPTER II

THE EFFECT OF ADRENALIN AND BETA-ADRENERGIC BLOCKAGE ON THE COAGULATION AND FIBRINOLYTIC SYSTEMS

Introduction.

Evidence accumulated since the beginning of this century has suggested that a link exists between the activity of the autonomic nervous system and the haemostatic mechanism. In 1903 Volburg and Richards injected adrenalin into the peritoneum of dogs and observed the appearance of a marked blood hypercoagulability. Cannon and Mendelhall (1914) confirmed and extended these observations in experiments in which the splanchnic nerve of dogs was excited by electrical stimulation, this being accompanied by a shortening of a modified whole blood clotting time. In further experiments it was proved that central nervous integrative functions could affect blood coagulability as shortened clotting times were obtained after cats were submitted to a severe stress. The common mediator for these neural induced coagulation changes was thought to be adrenalin.

The effects of adrenalin on the whole blood coagulation time was further investigated by Waldron (1950) and Forwell and Ingram (1957) and much of the earlier work was confirmed and extended. Marciniakowna (1957) infused adrenalin into animals and detected a rise of Factor VIII, and in 1961 Ingram demonstrated that the administration of adrenalin resulted in a rise of clotting Factor VIII, not only in the majority of human subjects, but also in some mild haemophiliacs. Similar findings were soon reported by

Egeberg (1963b); Goudemand et al. (1964); Naleczynska et al. (1966); and Libre et al. (1968).

It had been recognized also that blood coagulability could be affected by mental stress (Schneider, 1951; Dreyfuss, 1956; Friedman et al., 1958), and observations in patients with haemophilia have indicated that emotional disturbances contribute to the frequency of bleeding episodes (Browne et al., 1960). Variations in Factor VIII to mental stress have apparently not been reported in man but it is generally assumed, from adrenalin infusion studies, and the variations in Factor VIII that do occur in any one individual (Simone et al., 1967) that in vivo alterations of Factor VIII are possible in response to psychic stimuli.

The effect of adrenalin on fibrinolysis was not investigated until the studies of Biggs et al. (1947). Subsequent work adequately verified and extended their observations (Truelove, 1951; Kwaan et al., 1956; Sherry et al., 1959; Genton et al., 1961; Doni et al., 1963; Neri Serneri, 1965; Cash and Allan, 1967). It was also recognized that acute mental stress could affect fibrinolysis (Macfarlane and Biggs, 1946; Schneider and Zangari, 1951; Truelove, 1951; Ogston et al., 1962; Patsch, 1963; Ogston, 1964; Latner, 1967), and that this change was due to an alteration in the levels of circulating plasminogen activator.

Anatomical evidence for a role of the central nervous system in haemostasis was provided by Gunn and Hampton (1967). By electrical stimulation of certain areas of the brain in dogs, either increases or decreases of circulating Factor VIII could be obtained. Similar results have been reported by Correll (1968) in cats. Benetato et al. (1964), using ultra short wave

irradiation of the hypothalamic region in man, produced an increase in blood fibrinolytic activity, as did electric shock treatment in isolated dog's head preparations (Kowarzyk et al., 1962).

Thus, though the evidence was scanty there was enough experimental and circumstantial evidence to suggest that the autonomic and central nervous systems were in some way involved in the control of the coagulation and fibrinolytic systems. One important intermediary was adrenalin and thus much study has been directed towards its metabolic actions.

Some of the many physiological effects of adrenalin are thought to be mediated through two separate types of receptors, named by Ahlquist (1948) alpha and beta receptors. Drugs which block these receptors are referred to as adrenergic blockers and are listed below.

	Stimulated by	Blocked by
Receptors	Nor adrenalin Methoxamine Adrenalin	Phenoxybenzamine Dibenamine Phentolamine Tolazoline Dihydroergotamine
Receptors	Isoprenaline Adrenalin	Dichloroisoprenaline Pronethalol Propranolol BW61-43 H 56/28 ICI 50172

In general, alpha-receptors mediate vasoconstrictor responses, and beta receptors both vasodilator responses, as well as stimulation of cardiac rate and force of heart beat. Alpha adrenergic stimulators, such as noradrenalin and methoxamine cause peripheral arteriolar vasoconstriction and contraction

of the uterus, ureter, dilator pupillae, splenic capsule and pilomotor apparatus along with relaxation of the intestine, while beta adrenergic stimulators such as isoprenalin cause peripheral vasodilation, relaxation of the myometrium and bronchi, and augmentation of the rate and force of contraction of the myocardium.

Adrenergic receptors are also intimately involved in other metabolic processes of the body. Alpha and beta receptor stimulation by adrenalin results in a rise of free fatty acids (F.F.A.) derived from adipose tissue (Havel and Goldfien, 1959; Vaughan, 1961) and this increase is abolished by beta adrenergic blocking agents but not by alpha adrenergic blockers (Pilkington et al., 1962). The normal increase of blood sugar after adrenalin administration is blocked by the combined use of both alpha and beta blockers, but not by the individual blocking agents (Antonis et al., 1967).

Ingram and Vaughan-Jones (1966) investigated the effect of adrenergic blockers on the post adrenalin increase of Factor VIII. It was discovered that alpha blockade by phentolamine did not affect the increase of Factor VIII but beta blockade with pronethalol or propranolol completely blocked the rise. It was therefore assumed that the effect of adrenalin on Factor VIII was probably mediated by beta receptors. Another study in which the effect of an adrenergic blocker on the post adrenalin rise of Factor VIII was that of Ozag et al. (1966) who found that in animals there was a significantly lowered mean increase after the prior administration of the alpha blocker phenoxybenzamine.

The effect of adrenergic blocking agents on fibrinolysis was

investigated by Ishioka et al. (1967). Using equal doses they demonstrated that adrenalin did, and noradrenalin did not, shorten the euglobulin lysis time. They then assessed the effect of both alpha and beta blockade separately. It was found that the alpha blocker phenoxybenzamine did not modify the effect of the adrenalin induced fibrinolysis. On the other hand, the beta blocker pronethanol completely blocked the normal euglobulin lysis time response to adrenalin. The evidence thus suggested that the response of fibrinolysis to adrenalin was mediated in some way via beta receptors. On the other hand, Tanser and Smellie (1964) in four patients were not able to demonstrate any effect of pronethanol or BW 61-43 on fibrinolysis.

Project Aims.

This study was designed to answer various separate problems, some of which were related to the previous observations of Section 4:1 of this thesis, and others to the concept outlined in the introduction to this chapter.

In the first instance, it is possible that the exercise stimulus to the coagulation system (Section 4:1) was not of a sufficient degree to evoke a significant alteration in the circulating levels of Factor XII. It is known that an adrenalin injection or infusion will increase the levels of Factor VIII and is an easily controllable and relatively simple procedure. An investigation was therefore made to ascertain if Factor XII would respond more readily to an infusion of adrenalin. Other changes in coagulation parameters were simultaneously measured and included the alterations occurring

in Factor VIII and cryofibrinogen (H.P.F.).

An investigation was also made of the effects of adrenalin on the fibrinolytic system. Not only could the well documented changes in plasminogen activator be confirmed but the significance of this change could be assessed more readily by measurement of fibrin degradation products (F.D.P.).

Finally, the effect of beta adrenergic blockade on both the coagulation and fibrinolytic systems was more fully investigated. This study was designed to throw additional light on the modifying influences affecting plasminogen activator levels and particularly the relationships existing between adrenergic receptor sites and changes in haemostatic parameters. The physiological properties of a new beta adrenergic blocker drug (ICI 50172) were also studied and compared with Propranolol.

Subjects and Experimental Design.

A. Subjects.

Volunteers recruited for this study were medical colleagues, all of whom were accustomed to venepuncture and to intravenous infusion studies. All were in good health. Some of these subjects had taken part in previous investigations on the effect of adrenalin on fibrinolysis (Cash and Allan, 1967) and considerable experience had been gained on the technique and procedures associated with such infusions. The procedure was in general as outlined by Cash (1967).

Before the initial infusion it was explained carefully to each subject the nature of the test and possible symptoms which might arise during the course of the experiment were outlined. In all cases continuous

electrocardiographic recordings were made before, during and for a variable time following the infusions. The majority of the investigations were performed in the Department of Medicine procedure rooms, and two qualified medical personnel (plus technical staff) were in attendance throughout the duration of the experiment. Infusions were terminated immediately if any untoward symptomatology arose. On two occasions the infusions were discontinued; once when a subject developed marked bradycardia following a propranolol infusion, and once during an adrenalin infusion in a subject known to be susceptible to the frequent development of supra ventricular extra systoles. Neither of these two subjects were studied further. All other infusions were uneventful.

B. Procedures and Experimental Design.

The basic procedure was as follows, but was modified according to the experimental requirements.

After a supine rest period of 30 minutes a 20 gauge needle was inserted into a cubital vein and a resting blood sample obtained. After the removal of the syringe the needle was attached by means of an Anaesthesia Extension Set (Baxter - BR 335) to a 50 ml. syringe containing either saline or the adrenergic blocker drug already mounted in a Harvard Constant Infusion pump. The infusion was then commenced and continued for 10-20 minutes depending on the experimental plan. At the end of this time, the syringe was disconnected and the next blood sample obtained. The needle was then connected in a similar manner to a further 50 ml. syringe containing the adrenalin solution, and infusion continued for the planned time period.

Samples taken during the adrenalin infusion were obtained from the opposite arm, and to avoid an excessive number of venepunctures the needle was often left in situ and kept patent by a slow running 0.9% saline infusion. Final blood samples were taken during, at the end of, or following the infusion. These procedures were established as a smooth running routine thus obviating undue subject anxiety.

The adrenalin used was that of Parke-Davis (Batch number LFJ 308) each millilitre containing 1 mg. of adrenalin as the hydrochloride dissolved in normal saline with 0.5% chlorobutol as preservative. It was diluted with saline immediately before use to the required concentration. Ascorbic acid 3 mg./ml. was added as preservative for experiments which lasted for longer than five minutes.

The adrenalin dose infused varied with the experiment and was usually related to total body weight, and details are included in the relevant experiments.

The propranolol ("Inderal", ICI 45520) (L-isopropylamino-3-(L-nopthyloxy)-2-propanol hydrochloride) used was diluted in 0.9% saline to the required concentration and was infused over a time period of ten minutes. A syringe containing atropine 1.2 mg. was also drawn up to be used if severe bradycardia or hypotension developed. This was used on one occasion (vide supra).

Study I.

In this study, the effect of adrenalin with and without beta-adrenergic blockade on both the coagulation and fibrinolytic systems was assessed. The design of the experiment was similar to that of Ingram (1966)

and was as follows :-

1. Rest period 30 minutes.
2. First blood sample taken.
3. 0.9% saline or propranolol (1 mg./min) infused for 10 minutes.
4. Second blood sample taken.
5. Adrenalin (10-15 μ gm/min.) infused for 10 minutes.
6. Third and fourth blood samples taken; removed from opposite arm at 5 and 10 minute mark respectively.

Each subject was tested three times, at intervals greater than one week, twice with saline and adrenalin and once with propranolol and adrenalin. The following laboratory tests were performed on the blood samples so obtained.

1. Factor XII assay.
2. Factor VIII assay.
3. H.P.F. assay.
4. Euglobulin lysis time assay.
5. F.D.P. assay.

Results.

A. Factor XII.

The results in six subjects (nine infusions) are tabulated in Table 4:11. There was no increase in Factor XII levels during or following the adrenalin infusion. The mean 95% confidence limits of the Factor XII assays was 78 - 123% of the observed results.

TABLE 4:11

EFFECT OF A TEN MINUTE INFUSION OF ADRENALIN
(15 μ gm/min) ON THE LEVELS OF FACTOR XII.

Subject	Minutes		
	0	5	10
	% Factor XII		
J.D.	100	-	103
	100	-	92
S.D.	100	105	125
	100	-	85
L.F.	100	92	105
R.F.	100	-	115
P.D.	100	91	101
	100	-	119
G.W.	100	-	100
Mean	100	96	105

B. Factor VIII.

There was no detectable increase of Factor VIII levels after either the saline or propranolol infusions alone. The mean percentage increases of Factor VIII during and after the adrenalin infusion was 182% at 5 minutes, and 225% at 10 minutes (Table 4:12). The increase of Factor VIII post adrenalin was completely blocked by the prior administration of propranolol (Table 4:13).

TABLE 4:12

EFFECT OF A TEN MINUTE INFUSION OF ADRENALIN
(15 μ gm/min) ON THE LEVEL OF FACTOR VIII.

Subject	Minutes			
	-10	0	+5	+10
	% Factor VIII			
J.D.	-	100	-	150
	100	96	162	170
C.M.	100	100	143	172
	100	98	162	148
S.D.	-	100	-	330
	100	115	181	236
L.P.	-	100	-	367
	100	106	225	237
R.F.	-	100	-	296
	100	89	246	215
P.D.	100	103	173	208
	-	100	166	173
Mean	100	101	182	225

TABLE 4:13

EFFECT OF A TEN MINUTE INFUSION OF ADRENALIN ($15 \mu\text{gm.}/\text{min.}$)
ON THE LEVEL OF FACTOR VIII AFTER A PRIOR INFUSION
OF 10 mgm. OF PROPRANOLOL.

Subject	Minutes			
	-10	0	+5	+10
	% Factor VIII			
J.D.	100	96	108	100
C.M.	100	88	103	92
S.D.	100	100	92	115
C.F.	100	79	100	78
R.F.	100	110	110	90
P.D.	100	107	86	120
Mean	100	97	100	99

C. Cryofibrinogen (H.P.F.)

In twelve assays on eight subjects (Table 4:14) there was no significant alteration in the post adrenalin levels of H.F.P. ($t = 1.6599$, $p = N.S.$).

TABLE 4:14

EFFECT OF AN ADRENALIN INFUSION ($15 \mu\text{gm}/\text{min}/10\text{mins}$) ON THE
LEVEL OF CRYOFIBRINOGEN (H.P.F.) IN EIGHT SUBJECTS

Subject	Minutes			
	-10	0	+5	+10
	mgm/100 ml.			
1	-	30.3	-	31.8
	-	36.1	-	35.4
2	48.9	44.8	38.2	45.6
	-	56.3	-	60.4
3	51.0	51.6	-	54.2
4	39.8	38.7	-	43.7
	-	40.9	-	39.8
5	-	46.4	48.0	47.9
6	-	43.3	-	40.4
	-	53.0	-	61.0
7	-	45.0	-	44.8
8	-	54.6	-	53.6
Mean	46.6	45.1	43.1	46.6

D. F.D.P.

There were no consistent alterations in the levels of F.D.P. during or post adrenalin infusion in the subjects studied (Table 4:15). Propranolol did not appear itself to affect F.D.P. levels.

TABLE 4:15

EFFECT OF A TEN MINUTE ADRENALIN INFUSION
ON THE LEVEL OF F.D.P. IN FIVE SUBJECTS.

Subject	Minutes			
	-10	0	+5	+10
	F.D.P. μ gm./ml.			
1	-	15.0	-	18.3
	-	6.1	10.3	10.3
2	-	5.4	7.4	6.6
	7.8	8.4	6.5	6.5
	9.1	8.2	12.3	8.2
3	12.3	11.1	11.1	-
	6.6	6.1	6.6	7.3
4	-	13.2	-	12.2
	4.5	9.1	9.1	4.5
5	7.6	8.6	7.6	-
	-	15.2	9.2	7.2
Mean	8.0	9.7	8.9	9.0

E. Euglobulin Lysis Time.

There was a marked decrease in euglobulin lysis time during, and at the conclusion, of the adrenalin infusion (Table 4:16). The changes occurring at both the 5 and 10 minute period were highly significant when tested by a paired t test ($t = 10.4287$, $p < 0.001$; $t = 7.8305$, $p < 0.001$ respectively). After prior propranolol infusion, the fibrinolytic response to adrenalin was impaired (Table 4:17 and Fig. 4:3) but not completely blocked.

TABLE 4:16
EFFECT OF A TEN MINUTE INFUSION OF ADRENALIN
(15 μ gm./min.) ON THE EUGLOBULIN LYSIS TIME.

Subject	Minutes			
	-10	0	+5	+10
	E.L.T. in Minutes			
J.D.	-	508	-	100
	211	250	56	58
C.M.	378	307	75	75
	527	459	164	140
S.D.	-	179	-	34
	335	350	63	60
L.F.	-	61	-	21
	-	203	31	37
R.F.	-	258	-	45
	-	281	44	48
P.D.	-	142	-	40
	-	381	84	82
	483	460	61	75
Mean	387	295	72	63

TABLE 4:17

EFFECT OF A TEN MINUTE ADRENALIN INFUSION ON THE EUGLOBULIN
LYSIS TIME SUBSEQUENT TO AN INFUSION OF 10 mgm. PROPRANOLOL.

Subject	Minutes			
	-10	0	+5	+10
	E.L.T. in Minutes			
J.D.	413	412	210	168
C.M.	445	360	320	241
S.D.	745	666	488	341
L.F.	135	148	68	56
R.F.	250	232	-	130
P.D.	259	330	208	176
Mean	375	358	259	185

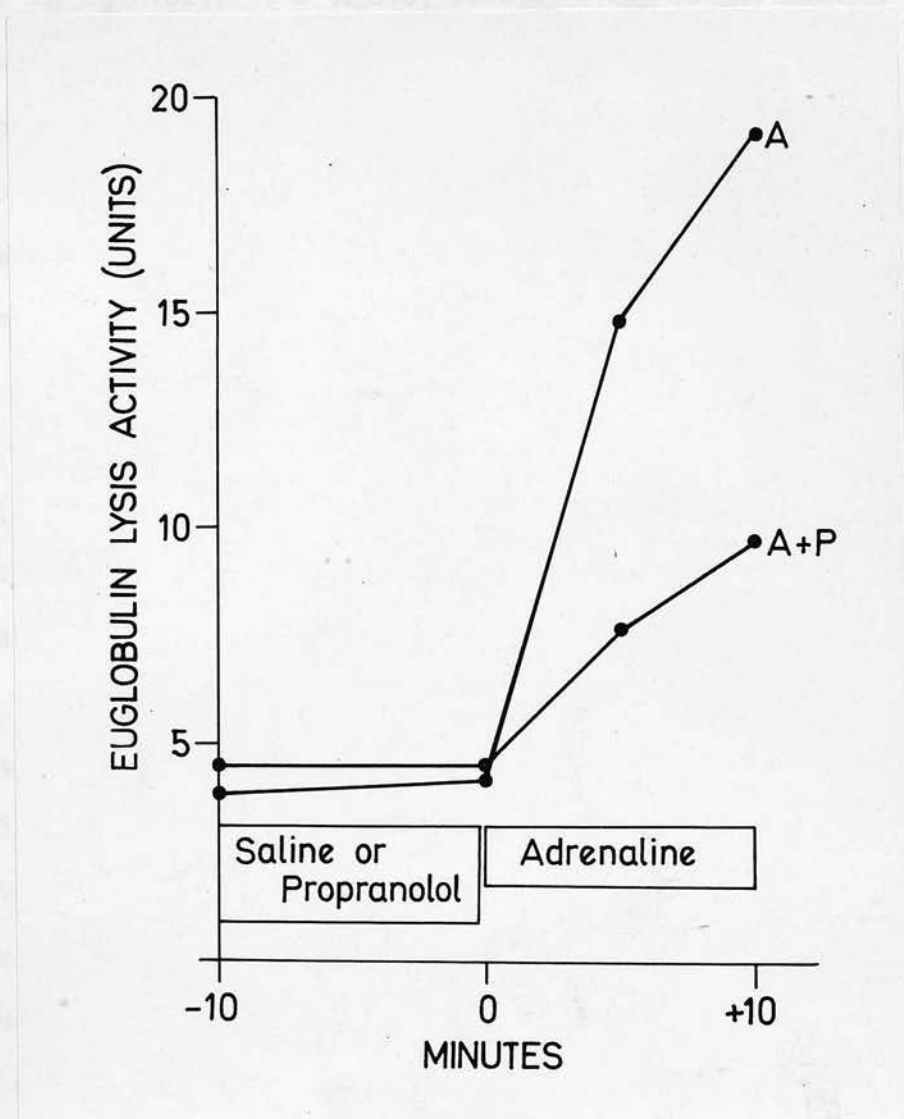


Fig. 4:3. Effect of a ten minute infusion of adrenalin on the euglobulin lysis activity.
(A - Adrenalin; P - Propranolol)

Comparison of the euglobulin lysis times at the 5 and 10 minute mark, with and without beta blocker, revealed that there was a statistically significant blockade of the plasminogen activator response to the adrenalin infusion ($t = 2.6743$, $0.05 < p < 0.025$; $t = 3.3010$, $0.025 < p < 0.02$, respectively).

The decrease of euglobulin lysis times after prior propranolol blockade was, however, still significant at both the 5 and 10 minute mark ($t = 4.1472$, $0.005 > p > 0.01$; $t = 4.5570$, $0.001 < p < 0.005$, respectively). Propranolol has therefore not completely blocked the post adrenalin increase in plasminogen activator levels.

Study II.

ICI 50172 (4-(2-hydroxy-3 isopropylaminopropoxy) acetanilide) is a new experimental beta adrenergic blocking drug. Animal work has indicated that it has a selective action, blocking catecholamine effects on the heart but not on the peripheral vascular tree. It appears to have no local anaesthetic action as has propranolol. In animals ICI 50172 will prevent the isoprenalin induced mobilization of free fatty acids although less strongly than propranolol. It is now under investigation at several centres to assess its clinical use in the treatment of cardiac arrhythmias.

In this study, the effect of adrenalin after prior blockade with this beta adrenergic drug, ICI 50172, on selected parameters of the coagulation and fibrinolytic system was evaluated and compared with that of propranolol. The degree of blockade achieved with this new drug was assessed separately by measurements of the changes occurring in response to adrenalin of free fatty acid levels. (The assay method used was that of Mosinger (1965), and these measurements were performed through the courtesy of Dr. M. Oliver)

The design of the experiments was as follows :-

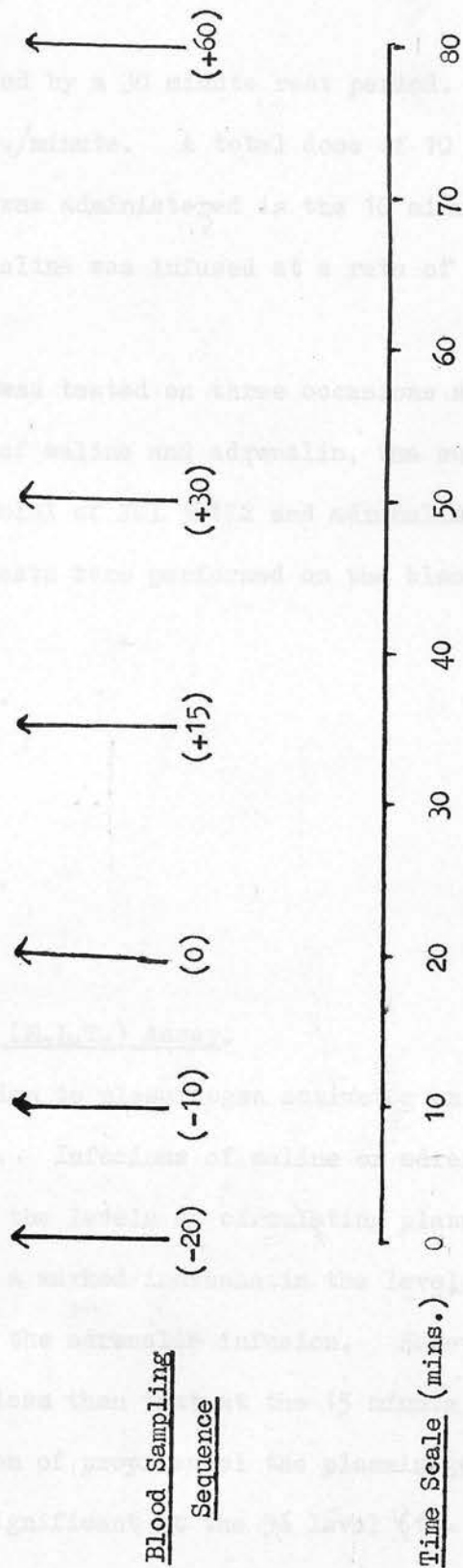
Expt. Type 1.



Expt. Type 2.



Blood Sampling
Sequence



Each experiment was preceded by a 30 minute rest period. 0.9% saline was infused at a rate of 5 ml./minute. A total dose of 10 mgm. of propranolol or 60 mgm. of ICI 50172 was administered in the 10 minute period of beta adrenergic blockade. Adrenaline was infused at a rate of 0.1 μ gm/kgm/minute.

Each of the five subjects was tested on three occasions at weekly intervals. The first infusion was of saline and adrenalin, the subsequent two infusions were of either propranolol or ICI 50172 and adrenalin.

The following laboratory tests were performed on the blood samples so obtained.

1. Euglobulin lysis time.
2. F.D.P. assay.
3. Factor VIII assay.
4. Free fatty acid assay.

Results.

A. Effect on Euglobulin Lysis Time (E.L.T.) Assay.

The results, after conversion to plasminogen activator units, are tabulated in Table 4:18 and Fig. 4:4. Infusions of saline or adrenergic blockers did not significantly alter the levels of circulating plasminogen activator. There was, as expected, a marked increase in the level of plasminogen activator in response to the adrenalin infusion. However, at the 30 minute mark the response was less than that at the 15 minute mark.

When preceded by an infusion of propranolol the plasminogen activator response was decreased, this being significant at the 5% level ($t = 2.0936$, $0.05 > p > 0.10$).

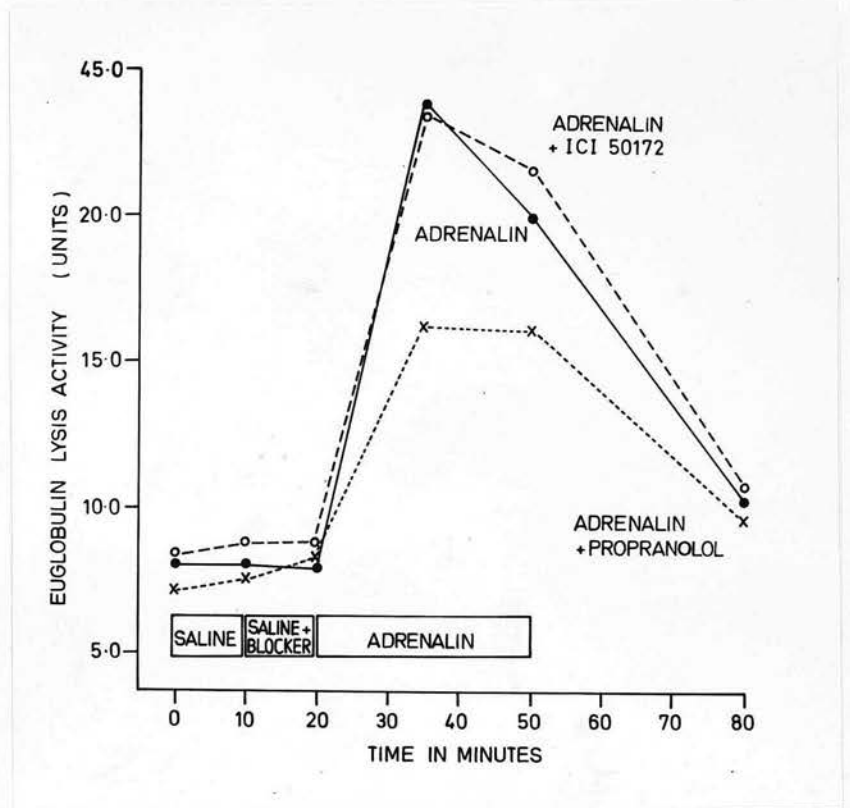


Fig. 4:4. Effect of a thirty minute infusion of adrenalin on the euglobulin lysis activity with and without prior infusion of Propranolol or ICI 50172.

TABLE 4:18.

THE EFFECT OF A THIRTY MINUTE ADRENALINE INFUSION, WITH AND WITHOUT PRIOR ADRENERGIC BLOCKADE,
ON PLASMINOGEN ACTIVATOR LEVELS.

	ADRENALINE						ICI 50172 + ADRENALINE						PROPRANOLOL + ADRENALIN					
	PLASMINOGEN ACTIVATOR UNITS																	
Subject	-20	-10	0	+15	+30	+60	-20	-10	0	+15	+30	+60	-20	-10	0	+15	+30	+60
S.M.	3.1	3.5	3.1	9.8	10.1	4.5	2.2	2.2	2.2	9.6	8.7	3.6	9.1	9.6	9.6	16.5	16.6	10.5
D.F.	14.5	14.6	14.4	31.7	27.1	18.5	16.2	17.5	16.4	36.1	32.5	20.6	-	8.3	10.1	18.6	20.9	12.0
D.B.	7.1	7.1	8.1	26.5	23.6	11.1	9.4	10.0	10.3	31.0	31.0	12.5	4.3	4.2	4.2	16.8	13.0	4.5
P.D.	8.1	7.8	6.3	26.5	14.9	4.1	8.5	7.6	8.1	21.0	19.1	6.5	9.3	9.3	10.4	17.1	17.3	10.3
J.C.	7.8	7.9	8.4	26.0	24.5	13.5	5.7	6.9	7.1	20.0	16.9	10.2	6.6	6.6	7.1	12.7	13.0	10.8
Mean	8.1	8.1	8.0	23.9	20.0	10.3	8.4	8.8	8.8	23.5	21.6	10.7	7.3	7.6	8.3	16.3	16.2	9.6
S.D.±	4.1	4.0	4.1	8.2	7.2	6.1	5.1	5.6	5.2	10.3	10.0	6.5	2.3	2.2	2.6	2.2	3.3	2.9

ICI 50172 however did not appear to modify the plasminogen activator response to adrenalin (Fig. 4:4).

B. F.D.P. Assay.

The results are summarised in Table 4:19. There were no detectable changes in the circulating levels of F.D.P. post adrenalin in the four subjects studied, this being in accord with the results observed in Study I. ICI 50172 had no effect on F.D.P. levels either with or without adrenalin administration.

C. Factor VIII Assay.

There was a significant increase of Factor VIII in response to the adrenalin infusions which was not significantly modified by the prior administration of ICI 50172. However, the increase of Factor VIII was completely blocked by the propranolol infusion, as in Study I (Table 4:20 and Fig. 4:5).

D. Free Fatty Acids (F.F.A.) Assay.

There was a highly significant increase of F.F.A. (Table 4:21) in response to the adrenalin infusion when the results were statistically evaluated by a paired t test ($t = 9.2436$, $p < 0.001$).

This response was blocked completely by propranolol but only partly by ICI 50172 (Fig. 4:6).

DISCUSSION.

The absence of a significant change in Factor XII levels after a ten minute infusion of adrenalin is similar to recent reports by Ratnoff (1966) and Naleczynska et al. (1966). As a relatively high dose of adrenalin

TABLE 4:19

F.D.P. LEVELS BEFORE, DURING AND AFTER A THIRTY MINUTE
ADRENALIN INFUSION, WITH AND WITHOUT A PRIOR INFUSION
OF ICI 50172

Subject	Minutes					
	-20	-10	0	+15	+30	+60
	F.D.Ps. (μ gm./ml.)					
ADRENALIN INFUSION						
S.M.	7.2	9.0	5.4	5.4	7.2	5.4
D.B.	6.3	7.2	7.2	7.2	9.0	10.8
D.F.	-	-	7.2	6.3	7.2	7.2
P.D.	-	5.4	5.4	5.4	5.4	5.4
J.C.	-	-	-	-	-	-
Mean	6.8	7.2	6.3	6.1	7.2	7.2
ICI 50172 PLUS ADRENALIN INFUSION						
P.D.	-	5.4	7.2	-	10.8	5.4
D.F.	10.8	9.0	7.2	10.8	7.2	7.2
D.B.	3.6	5.4	10.8	7.2	7.2	7.2
S.M.	10.8	8.0	10.8	10.8	10.8	7.2
J.C.	8.4	7.0	9.0	9.6	9.0	6.8
Mean	8.4	7.0	9.0	9.6	9.0	6.8

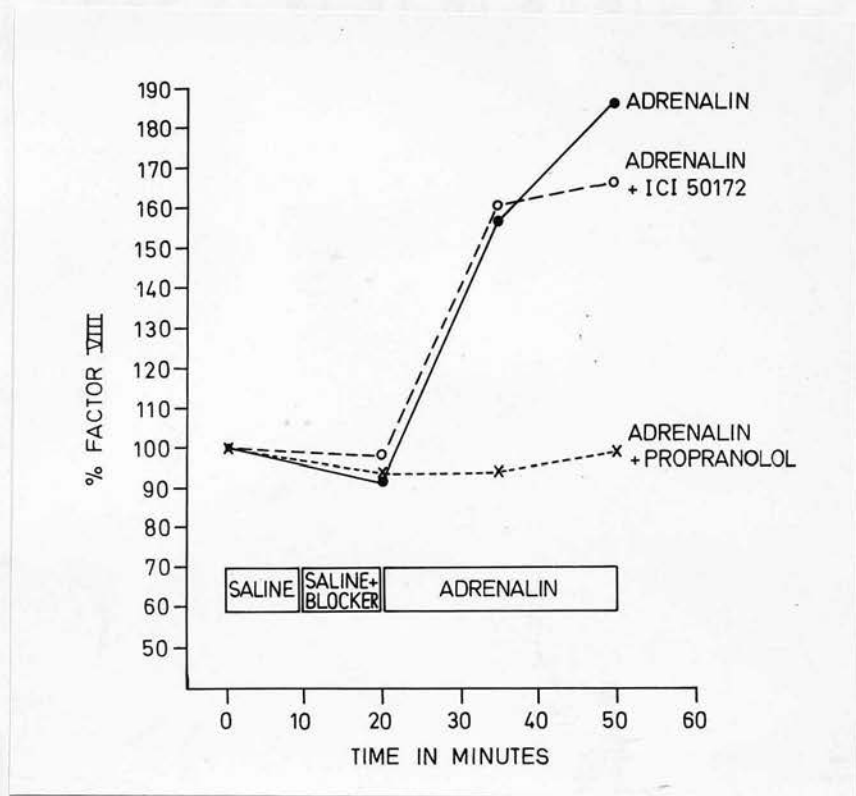


Fig. 4:5. Effect of a thirty minute infusion of adrenalin on Factor VIII levels with and without prior infusion of Propranolol or ICI 50172.

TABLE 4:20

THE EFFECT OF SALINE, ICI 50172 OR PROPRANOLOL, AND ADRENALIN
ON THE LEVEL OF FACTOR VIII.

Subject	ADRENALIN				ICI 50172 + ADRENALIN				PROPRANOLOL + ADRENALIN							
	Sampling Time (mins.)															
	-20	0	+15	+30	-20	0	+15	+30	-20	0	+15	+30	-20	0	+15	+30
S.M.	100	82	146	169	100	93	133	152	100	112	106	111	100	112	106	111
D.F.	100	102	190	227	100	91	153	154	100	90	81	87	100	90	81	87
D.B.	100	92	145	156	100	99	152	208	100	96	97	109	100	96	97	109
P.D.	100	80	144	212	100	109	141	135	100	83	94	95	100	83	94	95
J.C.	100	102	162	165	100	96	220	180	100	84	90	92	100	84	90	92
Mean	100	92	157	186	100	98	160	166	100	93	94	99	100	93	94	99
S.D.±	-	11	20	32	-	7	35	29	-	12	9	11	-	12	9	11

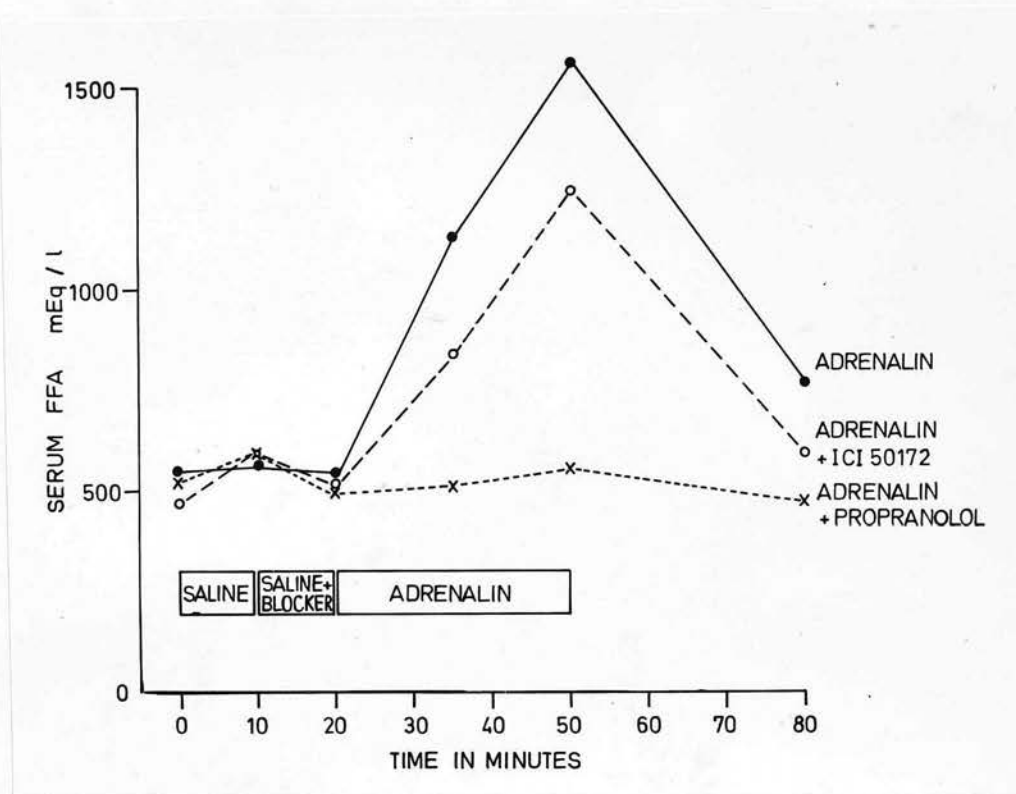


Fig. 4:6. Effect of a thirty minute infusion of adrenalin on the serum free fatty acid level with and without a prior infusion of Propranolol or ICI 50172.

TABLE 4:21

THE EFFECT OF SALINE, ICI 50172 OR PROPRANOLOL, AND ADRENALINE
ON THE LEVEL OF FREE FATTY ACIDS (mEq/L)

Subject	ADRENALINE						ICI 50172 + ADRENALINE						PROPRANOLOL + ADRENALINE					
	SAMPLING TIME (mins.)																	
	-20	-10	0	+15	+30	+60	-20	-10	0	+15	+30	+60	-20	-10	0	+15	+30	+60
S.M.	416	526	387	825	1131	569	376	576	520	680	1000	296	660	583	472	431	521	347
D.F.	582	649	664	1261	1881	1007	490	624	624	1060	1503	785	635	599	416	380	474	489
D.B.	492	500	400	1208	1658	775	474	533	526	883	1409	613	531	648	430	703	750	477
P.D.	752	664	704	1312	1704	992	625	617	586	869	1195	773	390	494	636	429	312	357
J.C.	417	523	556	1046	1430	497	394	606	354	709	1102	528	420	634	511	618	717	695
Mean	532	572	542	1130	1561	768	472	591	522	840	1242	599	527	591	493	512	554	473
S.D.±	141	78	146	198	289	234	99	37	103	153	209	201	122	60	88	140	180	140

(10-15 μ gm/min.) was used in the present study, it is unlikely that the lack of an increase was due to inadequate dosage. The absence of an increase of Factor XII after both exercise and adrenalin stimuli, using a widely accepted assay technique, casts further doubt on the previously accepted changes of Factor XII post exercise reported by Iatridis and Ferguson (1963).

There was no significant change of cryofibrinogen (H.P.F.) after the adrenalin infusion. This might indicate that mechanisms for generating cryofibrinogen in the exercise and adrenalin stress situations are quite different. The increase of cryofibrinogen post exercise is less likely to be dependent on the endogenous production of adrenalin and other unrecognized factors may be more relevant, such as the release of local tissue thromboplastins. Further elucidation is required of this interesting finding, particularly if more refined methods of measuring cryofibrinogen can be used.

The effect of adrenalin on circulating Factor VIII levels is similar to that of other investigators (see introduction). The individual response of Factor VIII to repeated adrenalin infusions varied, and this is probably a reflection of the inaccuracy of assay techniques when measuring high levels of a clotting factor. In general, subjects could be divided into either high, mid or low Factor VIII responders to the adrenalin infusion. Ingram and Vaughan-Jones (1966) in their experiments selected only those volunteers who consistently gave high responses. In the present study subjects were not selected on this basis.

In both studies it was confirmed that complete blockade of Factor VIII could be achieved by propranolol (Ingram and Vaughan-Jones, 1966). It has been suggested that altered circulatory dynamics may be the basis

for changes in Factor VIII elevations following adrenalin (Libre et al., 1968). The splanchnic circulation is probably influenced by adrenalin through both alpha and beta receptors (Price et al., 1967). Changes in blood flow through the splanchnic circulation and particularly through the spleen thus could occur under beta adrenergic blockade, decreasing the release of Factor VIII from this area. However, such an explanation is perhaps too simplified as it would not appear to explain the complete absence of a post adrenalin rise of Factor VIII after propranolol.

A post adrenalin increase in the level of circulatory F.D.P. could not be detected following either a ten or thirty minute adrenalin infusion in these studies. These results are at variance to preliminary observations previously reported from the laboratory (Das et al., 1967). However, the previous experiments were performed on sera removed after two minute infusions of lower doses of adrenalin, and these present studies are not directly comparable. Differences in assay techniques may also have occurred, perhaps with a decrease in discrimination of the assay, although this is less likely in view of the excellent sensitivity of the assay system in other concurrent studies. The differing results are not easily explainable but from the present experiments it is probable that if an elevation of F.D.P. does occur after an adrenalin infusion the rise is transient and certainly not great. Further studies using more sensitive techniques are required. Both propranolol and ICI 50172 alone had no consistent effect on levels of F.D.P.

The changes in response to an intravenous infusion of adrenalin of plasminogen activator levels have been noted previously (introduction). In the present experiments adrenalin produced a marked shortening of the

euglobulin lysis time. After thirty minutes of adrenalin infusion (Study II) there was a tendency for the lysis times to lengthen again (Fig. 4:4) and this may represent some exhaustion of the mechanism for producing plasminogen activator, or perhaps an increase of plasminogen activator inhibitors in response to adrenalin. These alternatives were not investigated but it would be of interest to measure the effect of a prolonged infusion (hours) of adrenalin on the euglobulin lysis time, coupled with an assessment of inhibitor levels. The exact mechanism for the appearance of plasminogen activator in the circulation after adrenalin administration is not known. Naleczynska et al. (1966) state that it is probably due to the release of a tissue activator of plasminogen into the blood stream. The observation of a significant correlation between the blood flow and plasminogen activator responses to adrenalin infusions could be relevant in this context (Cash et al., 1968). It is also possible that release of plasminogen activator from vessel walls is controlled, at least in part, by the sympathetic nervous system.

Both studies demonstrated that the administration of propranolol modified the response of plasminogen activator to the adrenalin stimulus (Figs. 4:3 and 4:4). These results are not in accord with Ishioka (1967) who was able to achieve complete blockade of the fibrinolytic response using the beta adrenergic blocker pronethalol. It is unlikely that the difference is explainable on incomplete adrenergic blockade as in the second study, using propranolol, the free fatty acid response to adrenalin was completely blocked as was the Factor VIII increase. The possibility exists, however, that not all receptor sites affected by beta adrenergic

drugs, show equal dose sensitivity. ICI 50172 had no detectable effect on the plasminogen activator response to adrenalin and in view of this it might be deduced that different beta adrenergic drugs, of varying chemical composition, may have differing actions on receptor sites. This could conceivably explain the variation in results between pronethalol and propranolol infusions, and it would be of interest to repeat the work of Ishioka (1967).

Although both the present results and those of Ishioka suggest that adrenalin stimulated changes in plasminogen activator may be mediated in part through the so-called beta receptors, the varying metabolic effects of separate beta drugs are not consistent with a simple classification of the adrenergic receptors into alpha and beta types. Much further doubt has been cast on this concept by the work of Himms-Hagen (1967), who regards the Ahlquist theory as untenable in view of the conflicting evidence obtained from both animal and human studies. Similar views have been noted by Epstein and Braunwald (1966).

In the present studies, the newly developed beta adrenergic drug ICI 50172 differed markedly in action from propranolol. Not only was it unable to block completely the post adrenalin increase in Factor VIII, but it modified only slightly the free fatty acid response. Although this may have been a dose effect, i.e. the amount of ICI 50172 infused may have been too low to affect these particular parameters, further increases in dosage were not regarded as justifiable, as in animals comparable doses had been able to produce adequate blockade (Fitzgerald, 1968). The effect of this drug therefore may be to divide beta adrenergic sites into at least two

groups - those affected and those not affected by ICI 50172 - thus even further complicating the simple concept of alpha and beta receptor sites.

It is clear from these preliminary observations that until much more is known of the exact nature of the so-called adrenergic receptor and its chemical reactions with blocking drugs, as well as the chemistry of reactions occurring at the cellular level, progress will not be rapid. However, this does not invalidate the basic premise of a regulatory relationship existing between the sympathetic nervous system and haemostasis and further studies, although difficult, will be of considerable interest.

SUMMARY.

The effect of adrenalin and beta adrenergic blockade in normal subjects was assessed by measurement of selected coagulation and fibrinolytic parameters.

There was a marked post adrenalin increase in the circulating levels of Factor VIII, plasminogen activator and free fatty acids. There was no change in Factor XII, cryofibrinogen or F.D.P. levels.

Prior beta adrenergic blockade with propranolol completely blocked the post adrenalin increase of Factor VIII and F.F.A., but only partially blocked the rise of plasminogen activator. With prior adrenergic blockade with ICI 50172 no effect on the post adrenalin increase of Factor VIII or plasminogen activator was noted, although there was a slight decrease in the maximum level of F.F.A. achieved.

The results are discussed briefly in the light of current concepts of adrenergic blockade and haemostasis.

COAGULATION, FIBRINOLYSIS AND PREGNANCY

Introduction

During the past few years, considerable attention has been directed towards the study of the changes in the coagulation and fibrinolysis systems during pregnancy. The purpose of this review is to summarize the present knowledge of these changes and to discuss their clinical significance. The changes in the coagulation system during pregnancy are well known and have been extensively studied. The changes in the fibrinolysis system during pregnancy are less well known and have been studied to a lesser extent.

SECTION 5

COAGULATION, FIBRINOLYSIS AND PREGNANCY.

The changes in the coagulation system during pregnancy are well known and have been extensively studied. The changes in the fibrinolysis system during pregnancy are less well known and have been studied to a lesser extent. The purpose of this review is to summarize the present knowledge of these changes and to discuss their clinical significance. The changes in the coagulation system during pregnancy are well known and have been extensively studied. The changes in the fibrinolysis system during pregnancy are less well known and have been studied to a lesser extent.

COAGULATION, FIBRINOLYSIS and PREGNANCY.

Introduction.

During the temporary, reversible physiological process of pregnancy, numerous alterations take place in the haemostatic mechanism. Not only is there a marked elevation of some coagulation factors, but fibrinolytic activity is significantly decreased, to such an extent that similar changes occurring in other circumstances might be considered abnormal.

This unusual biological situation could be of the utmost importance in our understanding of the control and mechanisms of coagulation and fibrinolysis. A clearer elucidation of these systems might provide relevant information in terms of the hypothesis of a dynamic equilibrium between coagulation and fibrinolysis, and clarify aspects of pathological haemorrhagic or thrombotic emergencies now known to occasionally complicate pregnancy.

Accordingly, a study was undertaken of selected facets of the haemostatic mechanism in pregnancy. A basic investigation of some of the reported changes in coagulation and fibrinolysis was first made, followed by additional studies of the reactivity of these systems to both exercise and surgical stresses. Finally, clinical instances of abnormal alterations in coagulation and fibrinolysis were diagnosed, investigated and treated and interpreted in the light of recent research developments.

CHAPTER I.

NORMAL LEVELS OF HAEMOSTATIC FACTORS IN PREGNANCY.

A. Introductory review of coagulation and fibrinolytic changes occurring in normal pregnancy.

The numerous alterations taking place in the various parameters of the coagulation and fibrinolytic systems in pregnancy, have been well documented. The changes described in coagulation include an increase in fibrinogen levels (Dieckmann and Wegner, 1934; Ratnoff and Holland, 1959; Pritchard and Brekken, 1967), and the concentrations of Factors VII and X (Loeliger and Koller, 1952; Alexander et al., 1956; Rutherford et al., 1964). There is no significant change in Factors II or V levels (Ratnoff et al., 1954; Pechet and Alexander, 1961; Kasper et al., 1964; Todd, 1965; Nossel et al., 1966). Some controversy remains regarding the levels of Factor VIII, but the most recent studies suggest that there is a significant rise (Strauss and Diamond, 1963; Kasper et al., 1964; Preston, 1964). With Factor IX levels, some investigators have found an increase, while others regard any changes as variable or not present (Koch, 1956; Ross, 1963; Todd, 1965; Nilsson and Kullander, 1967). Factors XI and XII have been less well studied, but are apparently unchanged or lowered in late pregnancy (Hilgartner and Smith, 1965; Nossel et al., 1966). Reports of Factor XIII levels are not available although Nossel et al. (1966) states that Factor XIII was present in all pregnant subjects investigated.

It has been known for some time that fibrinolysis, as assessed by

the various methods that measure plasminogen activator, is decreased in the latter part of pregnancy (Biezenski and Moore, 1958; Gillman et al., 1959; Biezenski, 1960; Shaper et al., 1965; Nilsson and Kullander, 1967). The apparent alteration in the fibrinolytic activity has been related to variations in levels of fibrinolytic inhibitors. Brakman and Astrup (1963) noted a selective increase in urokinase inhibitors in pregnancy and this finding was confirmed by Shaper et al (1968) and Kawano et al. (1968), although not by Nilsson and Kullander (1967). The latter authors did however detect an increase of antiplasmins and antiactivators in pregnancy. Guest (1954), Phillips and Skrodelis (1958) and Naidoo et al. (1960) were also able to find increased antifibrinolytic activity in pregnancy, but the specificity of the changes noted have not been clarified.

Few reports on the levels of plasminogen in pregnancy are available, but the consensus of opinion is that levels are slightly elevated (Mitchell and Cope, 1965; Brakman, 1966; Nilsson and Kullander, 1967). There is a fall in the platelet count throughout pregnancy (Shaper et al., 1968) and recent work has demonstrated that platelet adhesiveness does not alter (Nilsson and Kullander, 1967; Shaper et al., 1968).

B. Experimental study of the normal levels of selected coagulation and fibrinolytic parameters in pregnancy.

1. Subjects and Methods.

(a) Subjects.

The blood samples for these studies were collected at routine ante-natal clinics of the Simpson Memorial Maternity Pavilion.

Two studies were required for the investigation of F.D.P. in pregnancy. In the first study, specimens collected were divided approximately equally between trimesters. Blood samples were collected from 147 apparently healthy pregnant women. A further 21 samples were collected from non-pregnant pre-menopausal women.

In the second study, samples were similarly collected from 169 pregnant, and 43 non-pregnant women of a similar age group. The second study was necessary for technical reasons connected with the F.D.P. assay.

Subjects were selected at random, all were in good health at the time of examination, and without evidence of pre-eclamptic toxæmia. Most had had a short rest either seated or lying down immediately prior to venepuncture.

(b) Collection of blood samples and assay methods.

Venous blood was collected from an antecubital vein by the usual method. Excessive venous occlusion or traumatic venepunctures were avoided. Blood was collected into either a "citrate" tube, kept at 4°C (for plasminogen and euglobulin lysis time assays) and/or a "Trasylol-citrate" tube (for F.D.P. and/or fibrinogen assays) and/or a "Trasylol serum" tube (for F.D.P. assays).

The euglobulin lysis time assay was performed immediately; other plasma or serum samples were stored at -40°C until assayed. Each tube was given a code number and all technical staff were unaware of the clinical details of each subject.

- (i) The euglobulin lysis time and plasminogen assays were performed as previously described.
- (ii) The fibrinogen assays were performed either by the method of Ellis and Stransky (1961), or by the Thorpe heat precipitation assay (1968). Both gave similar results.
- (iii) The F.D.P. assay was as earlier described, apart from the following special arrangements.

In the first study which was run on serum prepared from blood collected into Trasylol-citrate, each trimester group was run separately. Samples were run at a single antisera concentration. Values above $20\ \mu\text{ gm/ml.}$ were repeated, but subsequent experiments demonstrated poor reproducibility related to the preparation of the Trasylol citrate specimens. The study was thus repeated.

In the second study on Trasylol serum samples, F.D.P. runs were carefully planned. The total number of specimens in each F.D.P. runs were divided approximately equally between the trimesters and the normal control sera specimens. Two concentrations of antisera were used in most instances. F.D.P. values above $20\ \mu\text{ gm/ml.}$ were repeated with good reproducibility. Additional fibrinogen standards were included as well as a standard control serum.

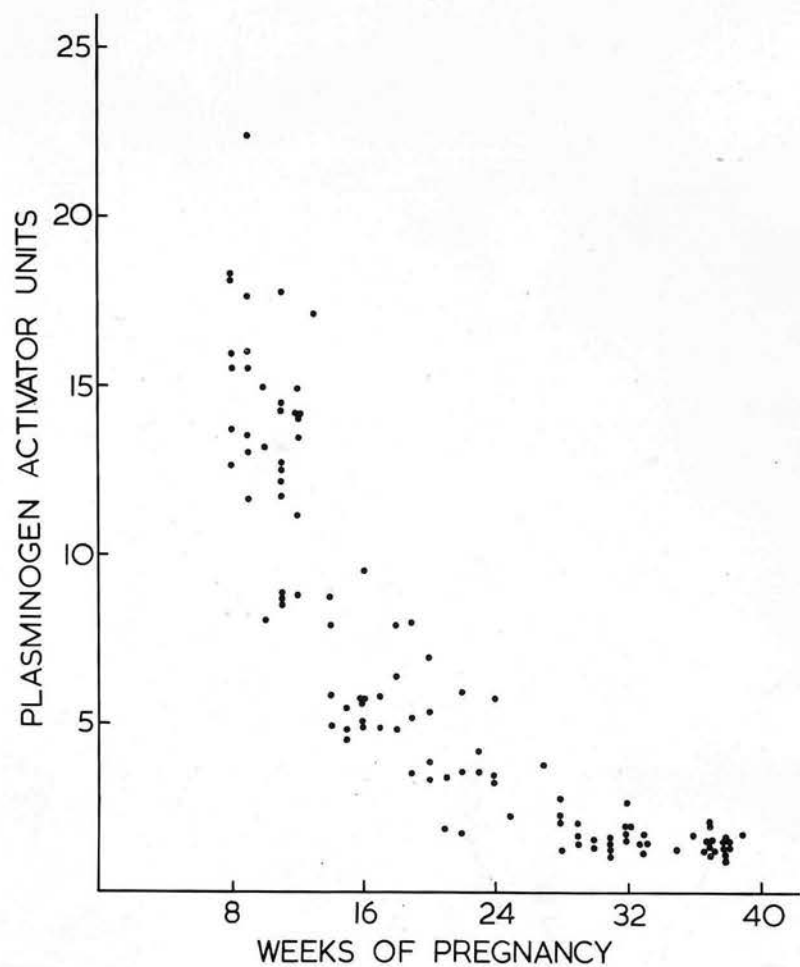
Both F.D.P. studies are reported; in the final analysis both gave

similar results. However, it was considered that the findings were of such import that the results of the first study required fuller confirmation. Absolute values vary between the two separate studies; this is because different fibrinogen standards were used for each study, which in turn affects both the calculated sensitivity of the assay system and hence absolute F.D.P. values. The values obtained in the second study are representative of those now obtained routinely.

2. Results.

(a) Euglobulin Lysis Time Assay.

A total of 111 assays of the euglobulin lysis activity were performed. These were divided approximately equally between the trimesters (1st trimester -40; 2nd trimester 33; 3rd trimester 38). The results after conversion to plasminogen activator units are depicted in Fig. 5:1 and Table 5:1. There is a gradual decrease in the circulating level of plasminogen activator throughout pregnancy, commencing at the 12th-16th week. Very low activator levels occur near the commencement of the third trimester, which are maintained until the termination of pregnancy. This "cross sectional" study of plasminogen activator levels throughout pregnancy is very similar to results obtained in a "longitudinal study" of nine pregnant subjects described in the following chapter of this thesis. The decrease in circulating plasminogen activator concentration is highly significant between the first and second ($t = 12.6866$, $p < 0.001$); first and third ($t = 22.876$, $p < 0.001$); and second and third



Plasminogen Activator levels throughout pregnancy
(III Observations.)

Fig. 5:1.

($t = 7.5953$, $p < 0.001$) trimesters of pregnancy.

TABLE 5:1
EUGLOBULIN LYSIS TIMES ASSAYS THROUGHOUT PREGNANCY
(111 OBSERVATIONS)

	1st Trimester	2nd Trimester	3rd Trimester
Euglobulin lysis time (Minutes) (Mean and S.D.)	100 ± 26 (33*)	336 ± 200 (42)	914 ± 191 (36)
Plasminogen Activator Units (Mean and S.D.)	13.7 ± 3.1	5.0 ± 2.8	1.5 ± 0.3
	$t = 12.6866$, $p < 0.001$		
	$t = 22.8767$, $p < 0.001$		

* Number of specimens.

(b) Fibrinogen Assay.

The results of 56 separate assays are depicted in Fig. 5:2 and Table 5:2. There was a highly significant increase of fibrinogen levels between the first and third trimester ($t = 4.0511$, $p < 0.001$). The increase was also significant between the first and second ($t = 2.7715$, $0.01 > p > 0.005$), and the second and third trimesters ($t = 2.5961$, $0.02 > p > 0.01$).

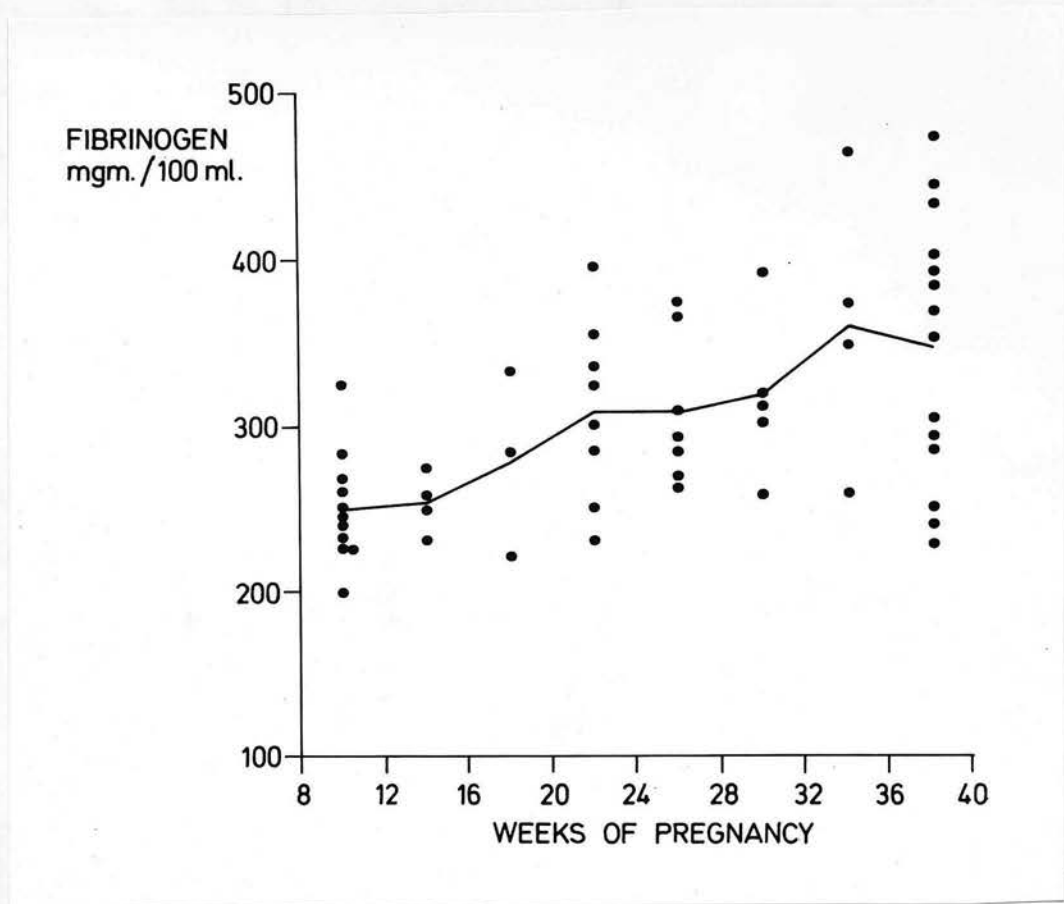


Fig. 5:2. Fibrinogen levels throughout pregnancy.

TABLE 5:2

FIBRINOGEN LEVELS THROUGHOUT PREGNANCY (56 ASSAYS)

1st Trimester	2nd Trimester	3rd Trimester
mgm/100 ml.		
250 ± 32 (11*)	296 ± 49 (22)	344 ± 74 (23)
$t = 2.7715, 0.01 > p > 0.005$		
$t = 4.0511, p < 0.001$		

* Number of specimens.

(c) Plasminogen Assay.

In 45 assays there was a significant increase of plasminogen levels throughout pregnancy (Table 5:3). The increase over the mean value of the first trimester was 20%. The increase in the third trimester as compared with the first trimester was highly significant ($t = 3.6631, p < 0.001$).

TABLE 5:3.

PLASMINOGEN LEVELS THROUGHOUT PREGNANCY (45 ASSAYS)

1st Trimester	2nd Trimester	3rd Trimester
C.U./ml.		
5.00 ± 0.81 (15*)	5.59 ± 0.87 (11)	5.98 ± 0.75 (19)
$t = 1.7692, 0.1 > p > 0.05$		
$t = 3.6331, p < 0.001$		

* Number of specimens.

(d) F.D.P. Assay.

The results of the first study are set out in Table 5:4, and of the second study in Table 5:4 and Fig. 5:3. In both studies there was a highly significant rise in the level of F.D.Ps between the first and third trimester ($t = 4.1895$, $p < 0.001$, and $t = 7.4213$, $t < 0.001$).

The same pattern was seen in each study between the control normal groups and the third trimester ($t = 2.9329$, $0.005 > p > 0.001$, and $t = 6.9313$, $p < 0.001$ respectively). In addition, in the second study, the rise between the first and second trimesters was significant ($t = 2.5672$, $0.02 > p > 0.01$). There was a wide scatter of F.D.P. values in both the second and third trimesters.

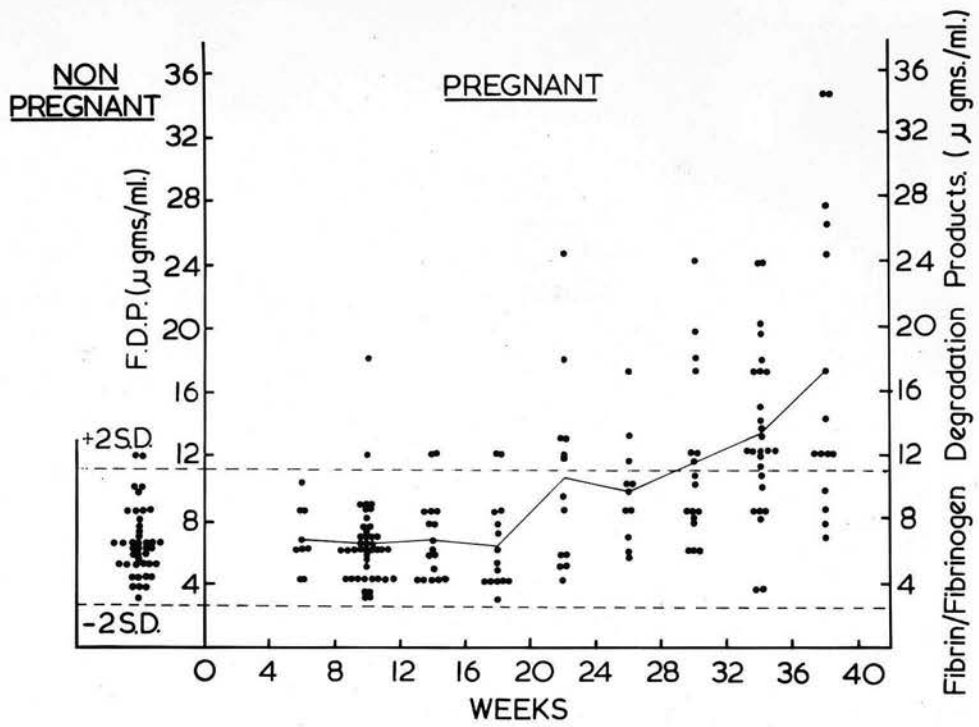
TABLE 5:4.

FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS LEVELS
THROUGHOUT PREGNANCY.

	1st Trimester	2nd Trimester	3rd Trimester	Control Group
		$\mu\text{gm/ml.}$		
1st Study (Mean and S.D.)	4.4 ± 2.7 (48*)	3.9 ± 2.4 (50)	6.9 ± 3.0 (49)	4.4 ± 3.6 (21)
2nd Study (Mean and S.D.)	6.6 ± 2.6 (53)	8.2 ± 4.1 (57)	14.0 ± 6.9 (59)	6.5 ± 2.1 (43)

* Number of specimens.

Euglobulin lysis time assays were performed on 36 of these samples, the selected specimens being evenly distributed throughout the



Fibrin/ Fibrinogen Degradation Products throughout pregnancy.

Fig. 5:3.

trimesters. There was no correlation between the observed euglobulin lysis times and the F.D.P. values ($r = 0.1085$, $p = \text{N.S.}$).

Fibrinogen assays were performed on 48 Trasylol citrated plasma samples, again evenly distributed throughout the trimesters. There was no correlation between the individual fibrinogen and F.D.P. values ($r = 0.0515$, $p = \text{N.S.}$) either within the complete group, or within trimesters.

In addition the available hospital and ante-natal clinic reports of the majority of subjects tested were reviewed. There was no significant difference in the mean ages of the groups of subjects tested. There was also no apparent correlation between parity, subsequent obstetrical complications encountered and the F.D.P. levels. In particular, higher levels of F.D.P. did not appear to be associated with haemostatic or thrombotic complications.

3. Discussion.

A decrease in the lytic activity of the euglobulin fraction of plasma was first detected by Gillman et al (1959). They noted that fibrinolytic activity decreased sharply between the 13th and 28th week of pregnancy. One year previously Biezenski and Moore, in a study of 260 subjects and using a clot lysis technique, demonstrated that an appreciable decrease of fibrinolysis was evident by the 16th - 20th week of pregnancy. In this present study of 111 subjects, using a sensitive euglobulinlysis time (ELT) technique for the measurement of plasminogen activator, it was demonstrated that changes in lysis time were detectable by the 12th - 16th week of pregnancy. The mean E.L.T. of the 40 subjects

in the first trimester of pregnancy was 101 minutes, with a range of 71 - 143 minutes. In a previously studied control group of 25 resting non-pregnant subjects (Cash, 1966), between the ages of 18 and 30 years, the mean E.L.T. was 154 minutes with a range of 68 - 595 minutes. The difference in mean values might be explained by the fact that the pregnant population samples were not taken while the subjects were necessarily in a resting state, but at a busy ante-natal clinic, where factors such as stress and exercise may have influenced the circulating levels of plasminogen activator. Notwithstanding, Biezenski and Moore observed that fibrinolysis was present in 93% of the control population but in every subject between 1 - 10 weeks gestation, and it is possible that marginal elevation of plasminogen activator levels may occur prior to the fall in the second trimester. Further controlled studies would be of interest, as little interest had been taken hitherto in changes occurring in early pregnancy.

There have been various explanations advanced for the decrease in lysis activity throughout pregnancy. The decrease could be due to increased levels of inhibitors as suggested by Biezenski and Moore (1958); and Gillman et al. (1959), but published reports on the behaviour of fibrinolytic inhibitors during pregnancy are still contradictory (Guest, 1954; Phillips et al., 1958; Naidoo et al., 1960; Biezenski, 1960; Correll and Sjoerdsma, 1962; Brakman and Astrup, 1963; Nilsson and Kullander, 1967; Kawano, 1968; Shaper et al., 1968). The relating of changes in plasma urokinase inhibitor levels to plasma activator concentrations is debatable as urokinase cannot normally be detected in peripheral blood (Kucinski et al., 1968), and this enzyme may be more sensitive to inhibition than plasma

activator (Brakman and Astrup, 1963). In the E.L.T. assay, fibrinolytic inhibitors are normally not precipitated with the euglobulin, but it is possible that very small amounts of inhibitors may be present. If this were a constant phenomena, and inhibitors did progressively increase in potency throughout pregnancy, the lengthening of the euglobulin lysis time could be explainable on this basis. However, we have no experimental evidence to support this theory, for in previous experiments no urokinase inhibitors could be detected in euglobulin precipitates from normal subjects. Also, inhibitors of fibrinolysis are normally present in plasma in excess of normal requirements and small increases in their absolute levels may not necessarily mean that there is greater inhibition of lysis activity. It is clear that until the fibrinolytic inhibitors are more precisely distinguished and their exact physiological role elucidated in relation to detected changes of circulating plasminogen activator, progress will be delayed.

It is unlikely that the decrease in euglobulin lysis activity can be attributed to substrate alterations in fibrinogen concentrations, as normal levels of plasminogen activator can be demonstrated post partum in the presence of even higher fibrinogen levels. There is also no correlation, at least in normal subjects, between variations in the euglobulin lysis times and plasminogen levels (Cash, 1966). Brakman (1966), regards absolute levels of plasminogen activator as being reduced in pregnancy and this concept is supported by Nilsson and Kullander (1967). If it could be proved that euglobulin was completely free of all fibrinolytic inhibitors the present results would also be consistent with this view.

The physiological mechanism by which lysis activity is reduced in pregnancy is not known. Theories advanced include changes in both adrenal and sex hormones (Gillman et al., 1959), and placental hormones (Shaper et al., 1966), although the part played by these substances in the control of normal fibrinolysis is not understood, as exogenous administration of derivatives of these hormones cannot duplicate the changes seen in pregnancy. However, it would be reasonable to suggest that the placenta could be intimately involved in the changes occurring in plasminogen activator. Not only do plasminogen activator levels increase soon after removal of the placenta (Shaper et al., 1966), but the placenta is rich in urokinase and trypsin inhibitors (Kawano et al., 1968), and does not contain, unlike most other organs, tissue activators. The study of fibrinolytic inhibitors produced by the placenta might therefore be of great interest as it is possible that one explanation of the low levels of plasminogen activator in pregnancy may be one of placental inactivation or neutralization of this enzyme. Plasminogen activator levels may be reduced because of excessive consumption in pregnancy, perhaps as part of a physiological mechanism to retain vascular patency in the heterografted placental bed. Also, little is known of other routes of excretion of plasminogen activator in pregnancy, such as by the liver or kidney, and whether excretion by such pathways is enhanced.

It is apparent that considerably more experimental work using monospecific tests are required if the significance and control mechanisms of this change in lysis activity is to be elucidated, although the measurement of F.D.Ps (vide infra) may be an important contribution to our understanding.

Reports of plasminogen levels during pregnancy are conflicting. Philips and Skrodelis, (1958); Ruckstuhl et al., (1962); Mitchell and Cope, (1965); Hedner and Nilsson, (1965); and Nilsson and Kullander, (1967), all found a moderate increase throughout pregnancy, while Shaper et al., (1965), and Brakman (1966) found no change. Some of the differences can be explained on methodology, as varying techniques have been used. However, in this laboratory, using a caseinolytic assay similar to that of Shaper et al. (1965), in a larger number of subjects, a small but significant increase in plasminogen could be demonstrated. It is possible this change is related to alterations in hormone levels occurring in pregnancy as administration of exogenous progestational agents can result in an increase of plasminogen levels. (Beller and Porges, 1967).

The increase in fibrinogen levels throughout pregnancy are well documented and the results presented here are similar. Pritchard and Brekken (1967), in a very large study of fibrinogen levels throughout pregnancy, found that the mean concentration increased about 50%; this is in agreement with the present study where the mean change as compared with a mean non-pregnant level of 225 mgm/100 ml. was 47%. Most studies have noted considerable individual variations and this is particularly evident in the wide spread of results in the third trimester. The plasma fibrinogen is elevated in many situations associated with physiological and pathological stresses (McKenzie et al., 1963) and the significance of its alteration in pregnancy is not known, although some writers have considered the change of teleological advantage in the control of haemorrhage at parturition.

Although fibrinogen levels have been intensively investigated by

other workers, it is of some importance that separate coagulation laboratories establish a normal range of fibrinogen values of pregnancy; not only do methods for measurement of fibrinogen vary with consequent differences in absolute values, but in some situations in pregnancy, a normal fibrinogen result may not exclude a pathological consumption of this protein.

It can be considered that the measurement of fibrinogen, plasminogen, plasminogen activators and inhibitors in pregnancy, provides only partial information on their dynamic interactions in vivo. Fibrinolysis has commonly been equated with plasminogen activator activity, but it is now increasingly apparent that such measurements may not necessarily be synonymous with in vivo lysis of fibrin. With the introduction of the F.D.P. assay, an entirely new approach can be made to the problems of in vivo lysis of fibrin, as it is assumed that the measurement of these degradation products represents the end product of dynamic in vivo reactions of coagulation and fibrinolytic factors. In the light of presently available evidence of a decrease in plasminogen activators and an increase of fibrinolytic inhibitors, it could be considered that actual lysis of fibrin would be decreased in pregnancy. To investigate this hypothesis a study of F.D.P. in pregnancy was made. Such a study has not previously been reported and the results noted in this thesis may be of considerable relevance to our understanding of the complex haemostatic changes occurring in pregnancy.

As can be observed from both sets of results (Table 5:4), F.D.P. levels increase throughout pregnancy. The finding of no correlation between the levels of serum F.D.P. and plasma fibrinogen in the pregnant group as a whole and in any one trimester indicates that the rise is not an artefact due to

unclothed fibrinogen remaining in the serum, but is a true reflection of the level of circulating, immunologically reactive F.D.P. Furthermore, it is not a simple reflection of plasminogen activator levels, as these decrease throughout pregnancy and no correlation could be found between activator concentration and the corresponding F.D.P. value.

One interpretation of these findings is that fibrinolysis is not decreased in pregnancy, but is in fact increased in the latter portion of the gestational period. Such a conclusion would require considerable qualification as the haemostatic changes occurring in normal pregnancy are complex, and, in the present state of knowledge, it would be hazardous to oversimplify such a system.

Most studies of fibrinolysis in pregnancy have centred on the measurement of blood plasminogen activator. The enzyme probably originates from venous and capillary endothelium (Todd, 1959) and is intimately linked with the intravascular lysis of fibrin (Sherry et al., 1959). The contribution tissue activators make to the circulating level of plasminogen activators has not been clearly defined.

On the other hand, F.D.Ps are an "end stage" in the proteolysis of fibrin and fibrinogen, and though circulating F.D.P. may represent only intravascular lysis, it is possible that they also partly represent active fibrinolysis in extravascular tissues. It is known that extravascular lytic activity can be considerable, for not only do most organs contain activators of plasminogen fulfilling important physiological roles in lysis associated with repair and inflammatory processes (Astrup, 1966), but fibrinogen is also present in an extravascular location (Gitlen et al., 1953)

and may represent 20 - 25% of the total body fibrinogen (Hammond and Verel, 1959; Lewis et al., 1961). Benz (1968) has been able to demonstrate extravascular lytic activity in tissues by the measurement of F.D.Ps. in both transudates and exudates, in the absence of degradation products in the simultaneously tested sera. Extravascular and intravascular lysis may therefore contribute to the circulating intravascular level of F.D.Ps. although definite evidence of this is lacking. This could also be one explanation of the poor correlation between the low observed levels of circulating plasminogen activator in pregnancy and the normal or elevated levels of F.D.P.

The uterus is particularly rich in tissue and vascular activators of plasminogen (Albrechtson, 1958). Mackay et al. (1967) have shown a marked arterio-venous gradient of plasminogen activator and F.D.Ps. across the non-pregnant uterus and suggest that fibrinolytic activity within the uterus is contributing to the systemic circulation. In pregnancy, with the greatly increased uterine vascularity, blood flow and tissue mass, the contribution of the uterus may be proportionately greater. Blood flow in the uterus increases from less than 100 ml./minute in the first trimester to 700 - 800 ml./minute at term (Metcalf et al., 1955; Assali et al., 1960; Huckabee, 1962). This could suggest that the uterus itself may be a source of circulating F.D.P. in late pregnancy.

It has not yet been defined clearly how F.D.Ps. are eliminated from the body but available evidence implicates hepatic and reticulo-endothelial (Gans and Lowman, 1967), and renal (Herschlein and Steichele, 1968) pathways. There is no evidence that renal function (Chesley and Chesley, 1939; El-Mahallarvi et al., 1968) or reticulo-endothelial

activity (McKay et al., 1964) is altered in normal pregnancy but there is some evidence that hepatic excretory function may be affected. Both Tindall (1965), using a modified bromsulphthalein excretion test, and Kessler and Aredos (1964), utilizing a battery of liver function tests were able to demonstrate small changes in liver metabolism in pregnancy. Simcock and Forster (1967) also found progressive cholestasis and impairment in mean hepatic excretory function. Delayed or slow excretion could possibly explain the increase in F.D.P., but this conclusion is unlikely for it has been shown that some patients with severe hepatic cirrhosis and a marked deterioration of all liver function tests, may have normal serum F.D.P. values (Das and Cash, 1968).

Earlier work in normal subjects has shown that exercise will increase the level of circulating F.D.P. in some individuals (Das et al., 1967). It could be assumed that the increased energy expenditure in attending the ante-natal clinic in the latter months of pregnancy could have led to a rise in F.D.P. However, there is no evidence that a fixed exercise procedure demands a higher energy expenditure in later pregnancy (Seitchik, 1967), and experiments to be described in the following chapter demonstrate that a significant rise in serum F.D.P. does not occur following moderate exercise in pregnancy.

Venous occlusion of the lower limbs occurs in normal pregnancy so that the femoral vein pressure gradually increases after the 20th week (MacLennan et al., 1943; Wright et al., 1950). Venous occlusion has been shown to increase the F.D.P. concentration in the occluded vessels (Nilehn et al., 1964), and it is conceivably possible that this could explain the

observed increase in circulating F.D.P. in late pregnancy. The other source of venous occlusion, i.e. that at the time of blood sampling, was reduced by removing all blood with minimal occlusion of the arm.

In some clinical and experimental hypercoagulable states there appears to be an increased turnover of fibrinogen (Izak, 1965). Studies of fibrinogen turnover in pregnancy do not appear in the literature and the only available information relevant to this topic is from Regoeczi (1968), who found the catabolic rate of fibrinogen was increased in one monkey in the third trimester. If fibrinogen turnover is increased in late pregnancy and assuming that the fibrinolytic system is, at least in part, involved in the metabolism of fibrinogen, it could be considered that this factor could explain the finding of increased F.D.P. levels. Further studies would be of considerable interest.

In a series of experiments on pregnant golden hamsters, Brown and Stalker (1968) have shown that following both spontaneous premature placental separation and the artificial application of trauma to the placenta, white emboli resembling fibrin emboli are released from the placental bed into the maternal and foetal circulations. If the damage is sufficiently severe, the animals develop a characteristic defibrination syndrome. In a recent report by Devi et al. (1968) attention has been focussed on the high incidence (64%) at term in normal pregnancies of macroscopic placental lesions in the form of Kline's haemorrhages, intervillous thrombosis, placental infarctions and retro placental haematoma. The presence of these lesions appeared to be associated with evidence of transplacental haemorrhage (foetal cells into the maternal circulation). Woodrow and Finn (1966) have

demonstrated a linear increase in the incidence of foetal cells in the material circulation during the third trimester. These observations, coupled with those of Brown and Stalker (1968) suggest that the incidence in circulating F.D.P. could be causally related to an increasing incidence of placental damage.

It is possible, although by no means proved, that the low levels of plasminogen activator in pregnancy are reflecting an increased consumption of this enzyme, subsequent to increased intravascular coagulation. Additional evidence for this could be the finding of a progressive fall in circulating platelet levels throughout pregnancy (Shaper, 1968). The present findings of increased F.D.P. levels would be consistent also, although the interactions between these various compounds is still far from clear and requires further elucidation.

It is unlikely that the significance of the elevation of F.D.P. in late pregnancy will be clarified until considerably more is understood of the metabolism and excretion of these products. However, despite these qualifications it is now possible to suggest that fibrinolysis is not necessarily decreased in pregnancy, as has been previously assumed. This reviewed concept of the place of fibrinolysis in pregnancy might go some way in explaining the relative infrequency of thrombotic episodes in the gestational period (Aaro, 1966) assuming, as would seem reasonable, that fibrinolysis has an important physiological function in removing intravascular fibrin associated with thrombi formation. The concept might also be incompatible with the theory that the increase of coagulation factors in pregnancy is a secondary effect of decreased fibrinolysis (Pechet et al., 1961).

4. Summary.

The changes in the intravascular concentrations of fibrinogen, plasminogen, plasminogen activator, and F.D.P. have been studied throughout normal pregnancy. The elevation of fibrinogen and plasminogen levels has been confirmed as well as the decrease in plasminogen activator concentration. In addition a new finding, that of increased F.D.P. with advancing pregnancy, has been presented and is further discussed in the light of the present knowledge of the coagulation and fibrinolytic systems.

CHAPTER 2.

THE EFFECT OF MODERATE EXERCISE ON THE FIBRINOLYTIC SYSTEM THROUGHOUT PREGNANCY

Introduction.

Earlier investigations from this laboratory, had established that the ability of a normal healthy individual to generate circulating plasminogen activator to a physiological stress such as moderate exercise was a reproducible phenomenon, that there was a significant difference between individuals and that it was possible to isolate a small sub-group whose fibrinolytic response appeared to be consistently impaired (Cash, 1966; Cash and Woodfield, 1968). This impairment was also evident following intravenous adrenalin and an exhaustive exercise procedure (Cash and Allan, 1967; Cash and Woodfield, 1967). Moreover, it was shown that in a population of 100 normal subjects, there was no correlation between the resting level of circulating plasminogen activator of an individual and his or her ability to respond. Thus on many occasions, subjects with low resting levels of plasminogen activator were observed, who subsequently responded well to the exercise and adrenalin stimuli. It was concluded that while these two aspects of in vivo fibrinolysis could have a complementary pathophysiological significance, their physiological control might be independent. In view of these findings and conclusions it was postulated that the ability of an individual to increase the level of circulating plasminogen activator might be of importance in clinical situations associated with acute focal

or disseminated intravascular coagulation and if so the so called "poor fibrinolytic responders" could be at risk (Cash, 1968).

These considerations suggested that an examination of the re-activity of fibrinolytic system in pregnancy, a condition known to be associated with unexpected episodes of intravascular coagulation would be of value, particularly in view of the already well documented work on Shwartzman type reactions.

The generalised Shwartzman reaction is one in which, following two suitably spaced injections of bacterial endotoxin or thrombin, animals develop a characteristic lesion of bilateral renal cortical necrosis (Thomas and Good, 1952). There is evidence to show that the renal lesion arises following the obstruction of the glomerular capillaries by fibrin thrombi subsequent to widespread intravascular coagulation (McKay and Shapiro, 1958; McKay et al., 1959; Bohle et al, 1959). There is also indirect evidence to show that one important protective mechanism acting against this pattern of events is the endogenous fibrinolytic response to the episode of intravascular coagulation (Margaretten et al., 1964, 1967; Phillips et al., 1968).

Pregnancy has been shown to "prepare" animals for the Shwartzman reaction, so that pregnant animals will succumb to a single injection of endotoxin or thrombin. There is suggestive evidence that a similar phenomenon can develop in the human species, for approximately two thirds of human cases of renal cortical necrosis occur in pregnant women (Duff and Murray, 1941). The complications of pregnancy in which they may arise are septic abortion, premature rupture of the membranes, abruptio placentae and

eclampsia, conditions all known to be associated with intravascular coagulation (McKay, 1964). It has been suggested that an important factor determining the state of "preparedness", and therefore a heightened susceptibility to Shwartzman type reactions in pregnancy, is the depression of fibrinolysis that occurs in the second and third trimesters (Biezenski and Moore, 1958; Gillman et al., 1959; Shaper et al., 1965; Nilsson and Kullander, 1967). This conclusion is based upon evidence obtained from pregnant women in the resting state and in view of the earlier observations, at least in non-pregnant human subjects, it cannot be assumed that their ability to effect a sudden increase in fibrinolytic activity following an appropriate stimulus is necessarily impaired. The present study was therefore designed to investigate this problem, using as a stress procedure a short treadmill exercise. In addition, the opportunity was taken to assess the response of other parameters to an exercise stress procedure. In vivo lysis of fibrin was assessed by the fibrinogen degradation products (F.D.P.) assay and the changes occurring in cryofibrinogen by measurement of heparin precipitable fraction (H.P.F.).

Subjects and Methods.

Volunteers for this study were recruited from a routine antenatal clinic of the Simpson Memorial Maternity Pavilion. Nine pregnant subjects were selected, aged between 22 and 32 years, mean 25.8 years. All were in good health and physical examination was normal in all cases. Both medical and obstetrical histories were found to be uncomplicated, and four subjects were multiparous. One subject (A.S.) developed mild

hypertension at the 33rd week and another (D.R.) a prolonged upper respiratory tract at 28 weeks, and studies at these stages were discontinued. Five of the subjects were followed throughout pregnancy, while the other four were investigated either in the second or third trimester. All were eventually delivered of live infants, and there were no major complications of note either at delivery or in the post partum period.

A degree of exercise was chosen which in preliminary experiments on 6 non-pregnant subjects (14 observations), produced a mean fibrinolytic response of 34%. The exercise was within the capabilities of all subjects even in late pregnancy, and the mean pulse rate increase was 62 ± 11 /minute. The procedure was repeated several times at weekly intervals, and then less frequently, until term. Venous blood was collected in the usual manner, both pre and post-exercise, from separate cubital veins.

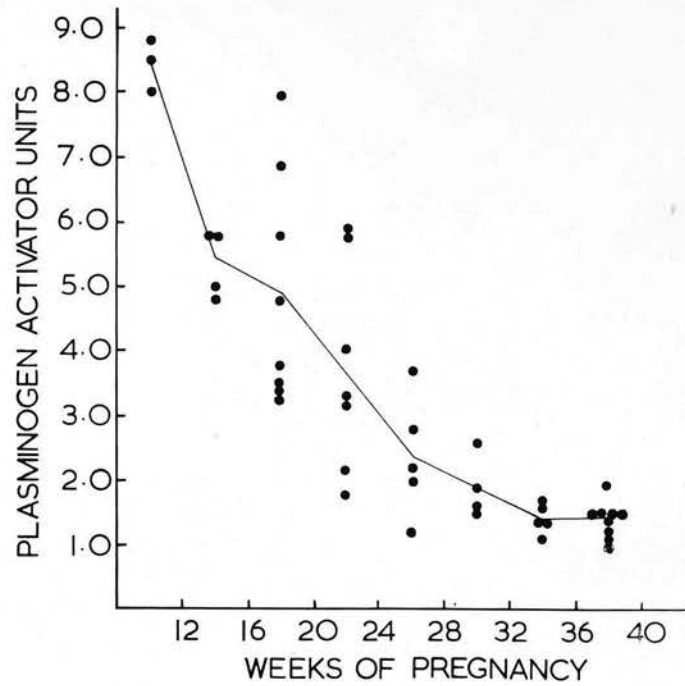
The fibrinolytic assay methods used were :-

- (a) The euglobulin lysis time.
- (b) Euglobulin and plasma drops incubated on human fibrin plates.
- (c) Fibrin degradation products assay.
- (d) Heparin Precipitable Fraction (H.P.F) assay.

The fibrinolytic response was calculated as previously described.

RESULTS.

The results of the resting euglobulin lysis time estimations in all subjects are summarised in Table 5:5 and Fig. 5:4. There was a gradual decrease of plasminogen activator levels throughout pregnancy with greatly lowered lytic activity in the latter half of the third trimester. Both



FIBRINOLYSIS IN PREGNANCY

Plasminogen activator levels throughout normal pregnancy. (9 subjects.)

Fig. 5:4

PROCEDURE,

Weeks	8 - 33 - 36			37 - 40		
Subject						
	B*	A	B	A	% Response	% Response
A.D.	-	-	16	739	22	-
M.L.	-	-	79	727	7	1
L.H.	-	-	-	-	-	4
A.S.	163	110	-	-	-	-
	153	80	-	-	-	-
	148	77	-	-	-	-
M.F.	-	-	30	1084	12	40
D.R.	-	-	-	-	-	-
A.B.	-	-	29	511	38	56
	-	-	-	-	-	35
E.D.	-	-	53	613	36	22
I.S.	-	-	55	602	8	25

TABLE 5:5.

RESULTS OF THE EUGLOBULIN LYSIS TIME ESTIMATIONS, BEFORE AND AFTER A MODERATE EXERCISE STRESS PROCEDURE,
THROUGHOUT PREGNANCY, IN NINE NORMAL VOLUNTEERS.

Weeks	8 - 12			13 - 16			17 - 20			21 - 24			25 - 28			29 - 32			33 - 36			37 - 40		
Subject	E. L. T. (M I N U T E S)																							
	B*	A	% Response	B	A	% Response	B	A	% Response	B	A	% Response	B	A	% Response	B	A	% Response	B	A	% Response	B	A	% Response
A.D.	-	-	-	-	-	-	343	250	27	742	395	47	1115	758	32	-	-	-	946	739	22	-	-	-
M.L.	-	-	-	-	-	-	372	141	62	400	310	23	600	397	34	804	670	17	779	727	7	1166	1152	1
	-	-	-	-	-	-	393	300	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L.H.	-	-	-	223	139	38	271	166	39	384	220	43	596	445	25	700	710	1	-	-	-	709	683	4
	-	-	-	260	155	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A.S.	163	110	33	-	-	-	163	116	29	227	144	37	465	285	39	505	387	23	-	-	-	-	-	-
	153	80	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	148	77	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.F.	-	-	-	270	172	36	223	163	27	410	252	39	643	393	39	-	-	-	1230	1084	12	870	524	40
	-	-	-	225	159	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D.R.	-	-	-	-	-	-	-	-	-	222	150	32	354	322	9	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	318	200	37	-	-	-	-	-	-	-	-	-	-	-	-
A.B.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	829	511	38	1093	484	56
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1310	857	35
E.D.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	876	443	49	953	613	36	884	686	22
I.S.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	655	602	8	870	650	25

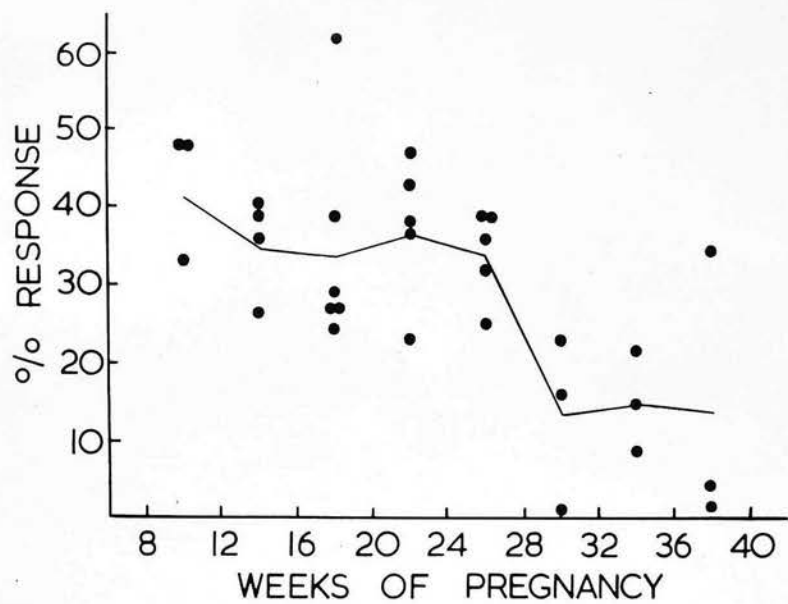
* Abbreviations B = Before Exercise
 A = After Exercise

euglobulin and plasma drops assayed on human fibrin plates showed a similar pattern. The decrease in activator levels between the 2nd and 3rd trimester was highly significant ($t = 7.498$, $p < 0.001$).

The percentage plasminogen activator response in all pregnant subjects to the exercise stress is also recorded in Table 5:1. In some subjects there was a progressive decrease in the fibrinolytic response with advancing pregnancy, while in others, the changes were less marked or absent. Fig. 5:5 illustrates the decrease of fibrinolytic response that occurring in the 5 fully studied subjects (A.D., M.L., L.H., A.S. and M.F.). The fibrinolytic response to the exercise stress, in these subjects, showed a highly significant decrease in the third as compared with the second trimester ($t = 5.102$, $p < 0.001$). In the 4 less completely studied individuals 2 showed no change in the fibrinolytic response and in 2 the response was diminished. There was no correlation, in the complete group of 9 subjects, between the resting level of plasminogen activator and the fibrinolytic response (Fig. 5:6). The changes in fibrinolytic response were also reflected in both the euglobulin and plasma drops on fibrin plates, although the difficulties in accurate assessment of small areas of lysis made this method of study less reliable than the euglobulin lysis time estimation.

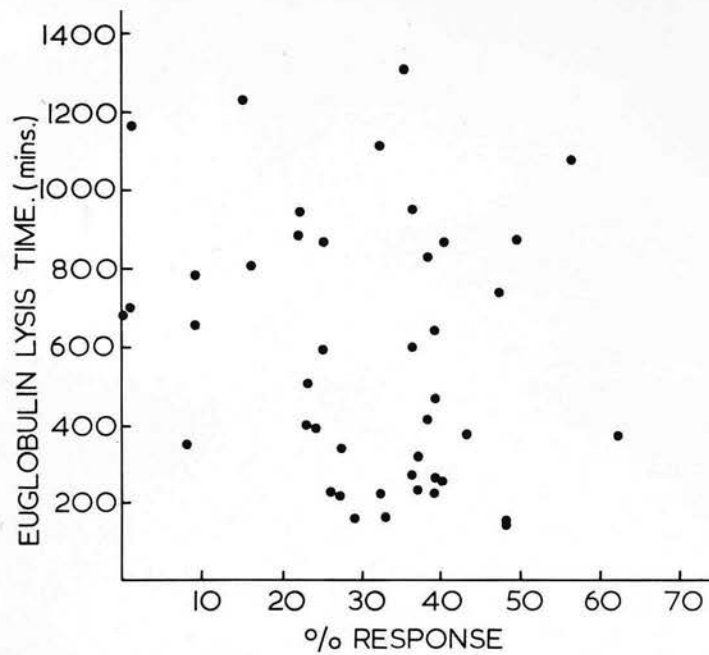
The results of the assays of fibrin degradation products are recorded in Table 5:6. In 36 observations in the 9 subjects at various gestation stages there was no statistically significant difference between the pre and post exercise means ($t = 0.7854$, $p = N.S.$). Furthermore, no subject showed a consistent change in the F.D.P. level on repeated testing.

Only a small number of H.P.F. assays were performed. There



Fibrinolytic response to moderate exercise throughout pregnancy. (5 Subjects.)

Fig. 5:5.



FIBRINOLYSIS IN PREGNANCY.

Correlation between Euglobulin Lysis Time and
Fibrinolytic Response to exercise. (43 observations.).
($r = -0.2345$; $p > 0.1$ N.S.; $y = 36.33 - 0.1x$).

Fig. 5:6.

TABLE 5:6

FIBRIN DEGRADATION PRODUCTS ($\mu\text{gms./ml.}$) ASSAYED BEFORE AND AFTER A MODERATE EXERCISE PROCEDURE IN NINE PREGNANT SUBJECTS (36 OBSERVATIONS)

Subject	Before	After	Subject	Before	After
F.D.P. $\mu\text{gms./ml.}$					
M. L.	7.2	14.4	L. H.	7.7	10.4
	10.8	5.4		14.4	14.4
	10.8	14.4		10.4	14.2
	14.4	7.2		Mean	10.8
	11.9	14.2	A. D.	5.4	10.8
	10.4	14.2		7.2	14.4
	14.4	10.8		14.4	7.2
I. S.	Mean	11.4		7.2	10.8
	17.8	14.2		14.2	14.2
	11.5	11.5		Mean	9.3
E. D.	Mean	14.7	A. B.	10.8	10.8
	5.8	11.5		14.4	14.4
	17.3	8.6		21.6	21.6
D. R.	Mean	11.6	A. S.	Mean	15.6
	10.8	7.2		4.3	5.8
	14.4	21.6		4.3	4.3
	10.8	10.8		2.2	5.8
M. F.	Mean	12.0		4.3	5.2
	5.4	14.4		7.1	5.0
	10.4	10.4		8.6	8.6
	10.8	7.2		8.6	8.6
	14.4	21.6		Mean	5.5
Mean	Mean	10.3	Mean	Before	After
	10.3	13.4		10.2	10.9

TABLE 5:7.

THE RESULTS OF H.P.F. ASSAYS, PRE AND POST MODERATE EXERCISE,
IN SEVEN PREGNANT SUBJECTS AT VARIOUS STAGES OF PREGNANCY.
(mgm/100 ml.)

Subject	Before	After	Gestation (wks.)
M. L.	42.4	69.3	14
	48.0	56.0	29
M. F.	28.7	33.9	16
	30.1	29.9	28
L. H.	43.3	47.4	16
	34.0	42.0	24
A. D.	19.0	45.1	21
A. B.	52.3	56.6	38
A. S.	36.7	45.6	24
D. R.	39.8	65.1	23
Mean	37.4	49.1	

was a post exercise increase in H.P.F. which when statistically tested by a paired t test was significant ($t = 2.9958$, $0.02 < p < 0.01$). (Table 5:7).

DISCUSSION.

A. Plasminogen Activator Baseline and Response Study.

The results of this study have confirmed the findings of other workers that the resting level of circulating plasminogen activator, as measured by the euglobulin lysis time and fibrin plates, is depressed in the latter months of normal pregnancy. However, an entirely new phenomenon - the development of a marked depression in the ability of some pregnant women to shorten the euglobulin lysis time following a standard exercise stimulus in the third trimester, has been observed. The good agreement between the euglobulin lysis time and fibrin plate assays during the periods of depressed response would suggest that the phenomenon is not related to changes in reactivity of plasminogen nor the increase in urokinase inhibitor levels described by Brakman and Astrup (1963); but that a defect had arisen in the production of plasminogen activator following the exercise stimulus. Unlike the marked depression of resting levels of plasminogen activator in the third trimester, a depression in response was not observed in all the subjects. This finding, as well as the absence of a significant correlation between baseline plasminogen activator levels and fibrinolytic response, appears to add further indirect evidence of a dissociation in the control mechanisms of these two fibrinolytic parameters (Cash and Allan, 1967)

The mechanisms of this change in fibrinolytic reactivity to exercise during pregnancy are unknown. The fibrinolytic response in any

one individual is related, in part, to the energy expenditure of the exercise procedure (Cash, 1967; Menon et al., 1967), so that the greater the energy expenditure the greater the fibrinolytic response. Few studies have been performed on the energy expenditure in pregnancy following a standard exercise procedure. Seitchik (1967) observed no difference between two populations of non-pregnant and pregnant women but noted that pregnant women, between 24 and 35 weeks, were the most efficient. Although it is tempting to use these findings to explain the results and although Seitchik's exercise procedure was sub-maximal, it was, however, a non-weight bearing procedure (bicycle ergometer) and, as he emphasised, the situation on a treadmill may be quite different.

It is believed that the release of plasminogen activator following such stimuli as exercise and epinephrine is a secondary passive phenomenon subsequent to vasoconstriction or vasodilation (Holemans, 1963). The peripheral blood flow in normal pregnancy, in terms of hand and foot blood flow, is increased. This vasodilation is believed to be due to a release of sympathetic vasoconstrictor tone, rather than from the direct effect of a circulating vasodilator humoral agent (Ginsburg and Duncan, 1967). Although Gibbon and Landis (1932) observed a further small increase in blood flow during indirect heating experiments it is conceivable that larger increases are impossible because vasodilation is near its maximum in the resting condition in pregnancy. Such a state of affairs could explain the depression in the fibrinolytic response to exercise in these experiments but does not explain why some subjects exhibited a normal response to the stress. It is interesting to note that the other occasion on which a

marked deterioration in fibrinolytic response to exercise has been observed, was in individuals during a period of severe mental stress (Cash and Allan, 1967) and Kelly has shown that severe mental anxiety may result in a 600 per cent increase in forearm blood flow due to intense vasodilation in the muscles (Kelly, 1966 and 1967).

Little work has been done on the measurement of the vascular response to physiological stimuli in pregnancy, but the work of Dolezal and Figar (1965), raises a further possible explanation of the findings. Using such stimuli as pain, cold and mental stress, they demonstrated that the vascular response of skin vessels in the hands during the first and second trimester was dominantly vasodilator and that in the third trimester most women displayed a vasoconstrictor response to these same stimuli. Mann et al (1967) have shown that in monkeys (*Macaca nemestrina*) the fibrinolytic response to drugs which produced a vasodilator response was ten times greater than that seen following vasoconstrictor drugs. It is conceivable, therefore, that the changes in fibrinolytic response during pregnancy may be a secondary effect of an altered vasoactive response, from dilation to constriction.

During pregnancy the concentration of circulating oestrogens and progesterone increases markedly (Short and Eton, 1959; Roy et al., 1963) and following delivery these fall precipitously, particularly oestradiol (Roy and Mackay, 1962). Although there is a certain degree of parallelism between the changes in concentrations of these hormones and the alterations in resting fibrinolysis there is no evidence that they are causally related (Shaper et al., 1966). Indeed, Bennet et al. (1966), found no significant

change in 8 male volunteers of the euglobulin lysis time following the administration of 3 mg. oestriol daily for 14 days. These results were confirmed by Brakman et al. (1967), who studied coagulation and fibrinolysis in both males and females, using oral contraceptive hormones. They also noted that a combination of progesterone and oestrogen increased resting spontaneous fibrinolysis and therefore did not reproduce the changes seen in normal pregnancy. Although the evidence at present is against a direct relationship between progesterone and oestrogens and the resting level of circulating plasminogen activator in pregnancy it is possible that they may influence the fibrinolytic response. Villadolid et al. (1965) have demonstrated that the conjugated oestrogens and oestriol succinate can impair the fibrinolytic response to intravenous adrenaline in adult male volunteers.

Evidence is now accumulating that certain organs, particularly the kidneys and uterus, may contribute to the maintenance of the circulating level of plasminogen activator (Buluk and Furman, 1962; Niewiarowski et al., 1964; Januszko et al., 1966; MacKay et al., 1967). Whether these organs play any part in the fibrinolytic response to a treadmill exercise procedure is unknown. Certainly, a variety of vasoactive agents, including adrenaline, produce a release of plasminogen activator from the kidneys of dogs (Holemans et al., 1965) and it is possible that this finding is also applicable to humans (Menon et al., 1968). Moderate exercise stimulates an increase in the concentration of plasma adrenaline (Von Euler and Hellner, 1952; Vendsalu, 1960), and it is possible that the kidneys are responsible, in part, for the elevation in the circulating level of plasminogen activator.

If this is so, then the observation of Epstein (1966) of diminished plasminogen activator in the endothelium of renal glomeruli in pregnant rats may be relevant and would partly explain our findings.

It is clear that until the fundamental mechanisms concerned in the release of plasminogen activator following stimuli such as exercise are understood, further progress in elucidating the cause of the deterioration in response in pregnancy will be delayed.

The clinical significance of a depressed fibrinolytic response to exercise in pregnancy must remain hypothetical. Certainly, there is no evidence that it is secondary to frank pathological changes in pregnancy - all the women, apart from one subject, studied in this investigation had an entirely normal pregnancy; all produced normal live babies, at term. It is possible that the condition of "preparedness" in pregnancy is more directly related to the ability to generate, rapidly, plasminogen activator to fibrinolytic stimuli. However, it is not known whether this depressed response is absolute, for Cash and Woodfield (1967) have already reported that some non-pregnant poor responders to a short sub-maximal exercise procedure will behave quite normally if the exercise is more prolonged. Furthermore, it cannot be assumed that the physiological type of response observed in the context of an exercise stress necessarily mirrors what might arise in pathological states of disseminated intravascular coagulation. It is also possible that, even in a state of impaired physiological fibrinolytic reactivity, copious quantities of tissue plasminogen activators may be released into the circulation, as in abruptio placentae. In such clinical circumstances - which may be quite unlike those seen in experimental

Shwartzman reactions - it is less likely that renal cortical necrosis and the associated sequelae would arise.

Despite these reservations, in the human species it is in pregnancy that the Shwartzman type of reaction is most commonly seen. The complications which give rise to this phenomenon are known to be associated with an uncompensated episode of intravascular coagulation. If the fibrinolytic enzyme system plays an important protective role in such circumstances, then the findings of a depressed responsitivity of the fibrinolytic system in the third trimester of pregnancy may be a crucial factor in determining whether the complications of intravascular coagulation are sustained, and in particular, renal cortical necrosis.

B. Fibrin Degradation Products Study.

The finding of no significant change in the circulating level of fibrin degradation products after exercise is similar to the earlier results following both moderate and exhaustive exercise procedures.

The coagulation changes occurring in pregnancy have been described as constituting a physiological hypercoaguability (Pechet et al., 1961); in such a susceptible milieu it might be expected that if a minor or perceptible degree of intravascular coagulation normally did occur in response to an exercise stimulus, fibrin formation and a compensatory lytic episode with the formation of F.D.P. could conceivably more easily take place. The negative findings in these experiments, do not however exclude this possibility. Firstly, any transient small change occurring may not be detectable by this type of assay. Secondly, the assay is sensitive to serum products of fibrinolysis which are plasmin resistant (Alkjaersig

et al., 1962), and not to the large molecular fragments released in the early stages of fibrin proteolysis by plasmin, which could conceivably be present in post exercise plasma (although this is unlikely, Sherry, 1967).

On the other hand, as earlier stated (Section 4:1), the plasminogen activator rise post exercise may be only a physiological mechanism to increase the potential for lysis if required. Actual lysis of fibrin in vivo may be quite unaltered despite the marked changes in activator levels. It is possible that a more severe exercise procedure may have provoked a change in the fibrin lysis rate, but this is unlikely in view of the previous studies on severe exercise (Section 4:1). From these results, however, it would appear that changes in levels of plasminogen activator within the physiological range, do not result in alterations in the degree of fibrin lysis in vivo.

C. Heparin Precipitable Fraction (H.P.F.).

These limited studies complement the studies performed on H.P.F. in both the moderate and exhaustive exercise procedures. There is again evidence of a significant rise in the post exercise level of H.P.F. and similar considerations apply to this finding as have been previously stated in earlier chapters. The number of observations is too small to comment on the degree of H.P.F. change; but it is of similar order to that of the moderate exercise experiments. No particular trend can be detected with increasing pregnancy.

These latter two studies do not provide definite evidence for or against the concept of a continuous fibrin formation-fibrin lysis mechanism

operative in vivo. It is quite possible that these two systems may be separately stimulated by physiological procedures such as exercise and only become linked when disseminated fibrin or plasmin formation exceeds a predetermined haemostatic mean. Further experiments using techniques such as assays of coagulation peptides and the estimation of cryprofibrin (Shainoff, 1960), may be able to demonstrate whether fibrin formation has commenced and these, coupled with a more sensitive assay for early and late degradation products of fibrin may aid in the clarification of these complex haemostatic changes.

Summary.

The fibrinolytic response to an exercise stress throughout pregnancy was studied in nine volunteers. There was a highly significant decrease in fibrinolytic reactivity in most subjects as well as a significant decrease in plasminogen activator levels. There was also no correlation between baseline plasminogen activator levels and the percentage fibrinolytic response. Fibrin/fibrinogen degradation products were not increased after exercise, but there was an increase in cryofibrinogen (H.P.F.).

The possible relevance of decreased reactivity of the fibrinolytic system is discussed in the light of Shwartzman-type reactions and it is suggested that a decreased fibrinolytic response to stress in the third trimester of pregnancy could be a factor in determining whether the complications of intravascular coagulation, and in particular, renal cortical necrosis, are sustained.

CHAPTER III

SURGERY, COAGULATION AND FIBRINOLYSIS IN BOTH PREGNANT AND NON-PREGNANT SUBJECTS

INTRODUCTION.

Increased fibrinolytic activity following surgery was first observed by Macfarlane (1937). Using a simple clot lysis technique he detected fibrinolysis in 75% of patients subsequent to surgery. Imperati, (1937) made similar observations and Macfarlane and Biggs (1946) attributed the changes to both the effects of mental stress and surgical trauma. Further studies, using more sensitive techniques revealed that an increase of fibrinolysis was particularly common during thoracic surgery (Mathey et al., 1950; Coon and Hodgson, 1952; Penn and Walker, 1954; Clifton et al., 1956; Ingram and Mann, 1959), and prostatic surgery (Scott et al., 1954; Lombardo, 1957; Ladehoff and Rasmussen, 1961). With the advent of cardiac bypass operations using extracorporeal circulations, interest in the fibrinolytic changes accompanying such surgery increased (Osborn et al., 1955; Von Kaulla and Swan, 1957; Nillson and Swedberg, 1959; Gans et al., 1961; Douglas et al., 1966) in an effort to identify and treat significant haemostatic problems. These investigations established that one main fibrinolytic change occurring in many different types of surgical procedures was a variable increase of circulating plasminogen activator (Anderson et al., 1962). Excess lytic activity could result in depletion of plasminogen and/or fibrinogen levels with or without bleeding complications. Lesser

degrees of fibrinolytic activation appeared to be of little pathological significance (Anderson and Mendelow, 1964).

Varied changes have also been reported in the coagulation system in response to a surgical system. Multiple or single defects of clotting factors had been detected (Cliffton et al., 1956; Karlson and Lerner, 1961) sometimes resulting in a bleeding diathesis. Minor alterations in the levels of circulating clotting factors are common and the changes can be attributed to their consumption in the clotting processes associated with surgery or to their proteolysis by fibrinolytic enzymes (Bergentz and Nilsson, 1961). Alterations in these factors however may be difficult to detect by conventional coagulation assay techniques although careful, systematic pre and post operation sampling will often reveal frequent diminutions of specific clotting factors, the extent of which will depend partly on the severity and site of the surgery (Gollub, 1964).

It is possible that some of the observed changes in fibrinolysis are a response to relatively minor degrees of intravascular coagulation occurring during the surgical process. Such a hypothesis would be difficult to prove or disprove conclusively, but one method of investigation would be to assess the end products of the clotting and lytic systems. If coagulation is activated with consequent fibrin formation, the lytic activity of the fibrin dissolving system might also be secondarily stimulated with fibrin lysis and the appearance of fibrin split products. The measurement of these products might not only reveal the significance of apparent changes in lytic activity occurring, but be an indirect measurement of the activity of the coagulation system.

Of no less interest are the alterations occurring in coagulation and fibrinolysis following surgery as the frequent occurrence of thrombotic phenomena still remains a major source of long term disability and appreciable mortality. Post-operation studies of coagulation have revealed a tendency to hypercoagulability (Gardikas et al., 1959; Egeberg, 1962; Davidson and Tomlin, 1963) associated with an apparent depression of fibrinolysis (Olow, 1963; Bennett et al., 1967). The significance of these changes in relationship to thrombosis is not known.

The project was thus designed to measure the changes occurring in selected parameters of coagulation and fibrinolysis in subjects submitted to an intra-abdominal operation, both in the operative and post-operative phase. To assess if pregnancy modified the fibrinolytic response of a subject to the stress of surgery, a group of subjects undergoing a Caesarian Section operation were also studied in a similar manner.

METHODS.

A. Selection and Management of Subjects.

Two groups of subjects were studied. The first group of patients were those admitted for a Caesarian Section operation with or without tubal ligation. The other group consisted of patients admitted for an intra-abdominal operation, usually a total or subtotal hysterectomy.

(a) Caesarian Section Operation Studies.

With the co-operation of the Obstetrical staff, Simpson Maternity Memorial Pavilion, selected patients were seen soon after their admission to the ward. In some cases, the admissions had been arranged in

advance; others were emergency admissions and details are given in the appendix. Each patient was interviewed, an explanation of the proposed study outlined and their permission and co-operation gained before blood sampling was commenced. It was possible to do this with some patients on the day prior to operation, but in others interviewing was done on the day of delivery. Most patients were very helpful and good liaison was achieved despite the repeated venepuncturing.

The mean age of the group studied was 30.8 years with a range of 20 to 40 years. The average stay in hospital (including pre-operative period) was 13.7 days. Complications occurring in the operative or post-operative period are detailed in the appendix or referred to in the relevant text. These Caesarian Section operations, which were often accompanied by a tubal ligation procedure, were usually completed within 45 minutes (range 30-65 minutes).

(b) Intra-abdominal Gynaecological Operation Subjects.

These patients were selected from the routine waiting list admissions of the gynaecological wards. Similar considerations apply to these patients as above. Most had been admitted for hysterectomy. All were premenopausal. Again, full details are given in the appendix.

The mean age of this group of women was 35.2 years, with a range of 21-43 years. The average stay in hospital, including pre-operative period, was 13.8 days. Case histories are referred to in the relevant text and appendix. The gynaecological operations were performed in an average of 53 minutes, with a range of 35-84 minutes.

B. Blood Sampling Methods.

In the Caesarian Section subjects it was planned to collect blood on (i) the day of admission; (ii) immediately prior to operation; (iii) as the umbilical cord was clamped; (iv) as the placenta was removed; (v, vi, vii and viii) 20, 40, 60 and 120 minutes after removal of placenta; (ix, x and xi) on days 1 and 2 post operation; and thereafter on alternate days.

In the gynaecological subjects, samples were to be obtained on (i) the day of admission; (ii) immediately prior to operation; (iii, iv, v and vi) 20, 40, 60 and 120 minutes after commencement of the operation; (vii, viii and ix) on days 1, 2 and 3 post operation; and thereafter on alternative days.

It was not possible to sample at all these points. The reasons for this were varied. In a few patients in the post-operative period, venepunctures were discontinued because of undue patient stress. Other investigatory procedures commonly precluded venepuncture on the planned day. On occasions, despite good liaison with the clinical staff, the patient had already been venepunctured. Some patients felt too unwell to co-operate, while others developed complications such as chest infections and anaemia; in these situations, venepuncture was not always performed. Some were discharged or transferred from hospital relatively soon after operation. Technical laboratory considerations also precluded sampling on a few occasions.

In the operative period, multiple blood samples were usually obtained from a cubital vein catheter inserted prior to operation. In

a few patients, separate venepunctures on varying veins were performed and minimal venous occlusion was used on all occasions.

In the post-operative period, samples were obtained by venepuncture from the least damaged vein after ensuring, wherever possible, that the patient had been resting for at least 30 minutes prior to sampling.

The volumes of blood collected were kept to a minimum compatible with laboratory test requirements. In addition, the volume of blood collected at each sampling point was varied according to the requirements of the assay.

C. Assay Techniques.

(a) Euglobulin Lysis Time.

This assay was performed immediately and specimens were not kept at 4°C for longer than 10 minutes before centrifugation.

(b) Fibrinogen Assay.

Blood, collected into a tube containing both trasylol and sodium citrate, was centrifuged, the plasma separated and stored at -40°C until assayed by the Ellis-Stransky method.

(c) Fibrin/Fibrinogen Degradation Products Assay.

Blood was collected into a glass tube containing trasylol. Serum was stored in duplicate plastic tubes at -40°C until assayed by the previously described method. All specimens from an individual patient were assayed in one large single assay, and high values (above 50 µgm/ml.) were repeated. Controls were as previously described.

RESULTS.

A. Gynaecological Operations.

(a) Euglobulin Lysis Time Assay.

The complete results are summarized in Table 5:8 and Fig.5:8.

It can be seen that there was a marked increase in euglobulin lysis activity occurring during the operative period, with a fall below the pre-operative level in the days following surgery.

There was a slight increase of euglobulin lysis activity in the blood specimen removed on the day of operation as compared to that removed on the day prior to surgery, but the change was not significant when assessed by a paired t test ($t = 1.0271$, $0.4 > p > 0.3$).

The alteration in euglobulin lysis activity reached significant levels within 20 minutes of commencement of the operation ($t = 2.8933$, $0.01 < p < 0.005$). At the 40 and 60 minute stage the increase was highly significant ($t = 3.8311$, $p < 0.001$; $t = 5.2877$, $p < 0.001$ respectively) but by 120 minutes, the difference was no longer significant ($t = 1.6688$, $0.2 < p < 0.1$). The change of lysis activity was therefore a transient one. In the post-operative period, the decrease of euglobulin lysis activity, as compared with the pre-operative level was significantly lowered until the 7th to 8th days. The significance levels are summarised in Table 5:9.

(b) Fibrinogen Assay.

The fibrinogen assay results are tabulated in Table 5:10 and charted in Fig. 5:9.

There was no significant difference between the pre and

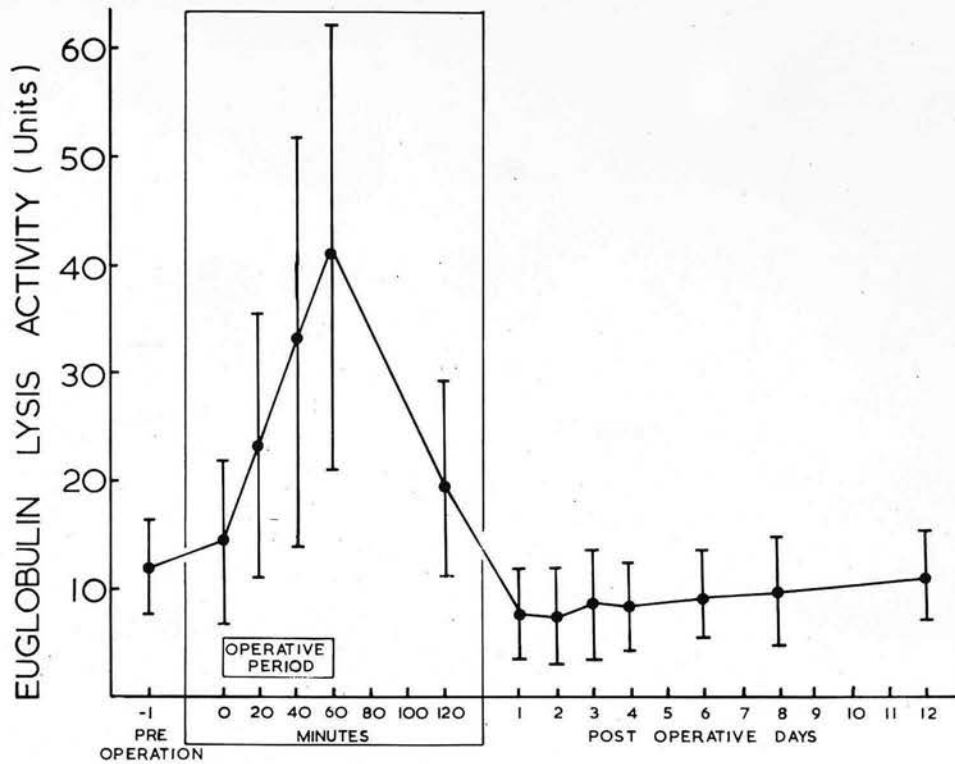


Fig. 5:8. The effect of an intra-abdominal gynaecological operation on the euglobulin lysis activity prior to, during and following surgery.

TABLE 5:8.

FIBRINOLYSIS (EXPRESSED IN EUGLOBULIN LYSIS ACTIVITY UNITS) PRIOR TO,
DURING, AND FOLLOWING AN INTRA-ABDOMINAL GYNAECOLOGICAL OPERATION IN TEN SUBJECTS

Subject	Pre-Operation		Operation Period - Minutes					Post Operation Period - Days											
	B ₁ **	B ₂ **	10*	20	40	60	120	1	2	3	4	5	6	7	8	9	10	11	12
H 1	18.6	26.0	27.0	24.0	25.0	68.4	22.4	2.2	2.2	2.1	-	-	1.8	-	3.1	-	7.4	-	-
2	-	10.0	-	26.5	-	54.1	-	8.8	5.8	6.1	-	8.7	-	5.3	-	-	-	-	-
3	16.0	9.2	-	22.4	-	27.0	26.5	9.0	14.1	-	6.6	-	7.6	-	9.0	-	-	-	-
4	11.5	13.2	12.4	17.1	24.0	28.2	25.4	7.5	-	7.8	-	14.6	-	7.9	-	-	15.8	-	-
5	-	29.5	-	29.5	41.9	76.4	-	14.4	1.8	15.6	7.8	12.0	-	13.8	-	18.0	-	-	-
6	-	8.7	-	-	-	43.3	6.9	4.3	4.5	4.4	1.4	-	-	6.4	-	-	10.2	-	-
7	15.4	21.6	17.8	20.9	24.1	15.2	-	7.7	12.3	-	15.2	-	13.5	-	12.6	-	8.3	-	-
8	7.3	15.2	13.8	14.2	21.3	18.0	19.7	1.8	9.3	-	6.6	-	5.0	-	-	-	-	1.8	-
9	11.8	7.9	-	-	-	43.3	7.8	2.2	2.2	10.8	9.6	-	-	8.7	-	-	-	10.0	-
10	6.5	15.3	-	31.7	72.2	65.0	30.2	13.4	14.1	14.4	-	6.8	-	21.3	-	12.7	-	13.1	16.2
Mean S.D.	12.4 ±4.5	15.7 ±7.6	21.4 ±6.5	34.8 ±19.8	43.9 ±21.7	19.8 ±9.1		7.1 ±4.5	7.4 ±5.2	8.7 ±5.1	7.9 ±4.5	8.8 ±4.4		9.8 ±5.4			11.4 ±4.8		

* Times after commencement of operation

** B₁ = Day prior to operation

B₂ = Immediately prior to operation

TABLE 5:9.

SUMMARY OF SIGNIFICANCE TESTS (PAIRED t TESTS) OF CHANGES
IN EUGLOBULIN LYSIS ACTIVITY FOLLOWING SURGERY.

Euglobulin Lysis Activity			
Pre-operation	: Post-operation	Paired t Test	
Pre-operation	: Day 1	3.0631	0.005 > p > 0.001
Pre-operation	: Day 2	2.7594	0.01 > p > 0.005
Pre-operation	: Day 3	2.0141	0.1 > p > 0.05
Pre-operation	: Day 4	2.2226	0.5 > p > 0.025
Pre-operation	: Days 5 + 6	2.1788	0.05 > p > 0.025
Pre-operation	: Days 7 + 8	1.7762	0.1 > p > 0.05
Pre-operation	: Days 9 - 12	1.2494	N.S.

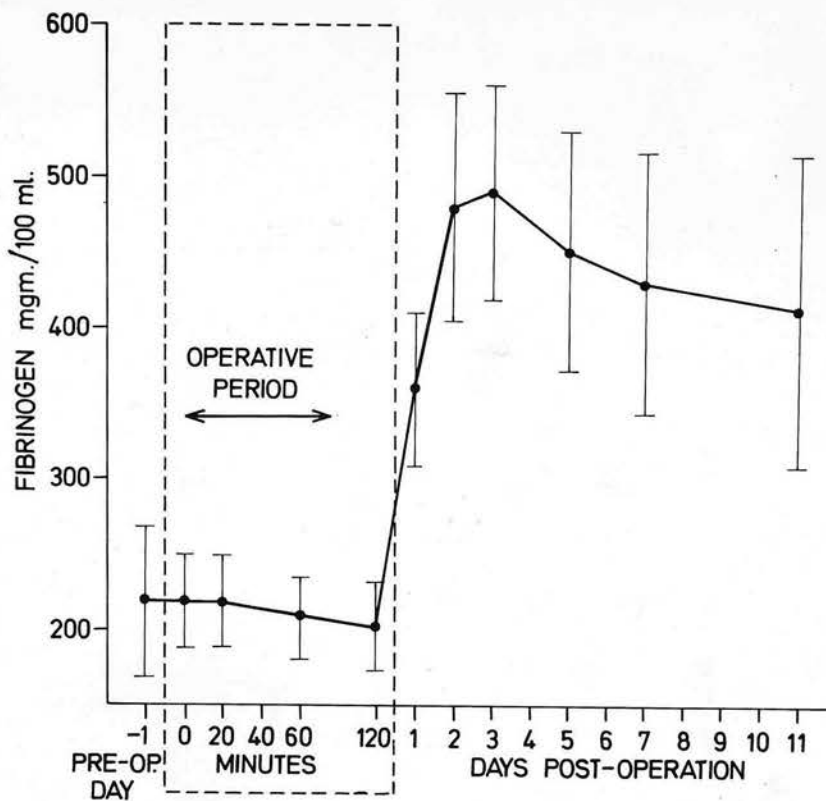


Fig. 5:9. Fibrinogen levels prior to, during and following an intra-abdominal gynaecological operation.

TABLE 5:10.

FIBRINOGEN LEVELS (mgm/100 ml.) PRIOR TO, DURING, AND FOLLOWING
AN INTRA-ABDOMINAL GYNAECOLOGICAL OPERATION IN TEN SUBJECTS.

Subject	Pre-Operation		Operation Period				Post Operation										
	**B ₁	**B ₂	Minutes				Days										
			20*	40	60	120	1	2	3	4	5	6	7	8	9	10	11
H 1	283	264	274	283	250	244	441	515	607	-	-	562	-	548	-	548	-
2	-	-	214	-	225	-	350	444	451	-	451	-	437	-	-	-	-
3	194	196	191	-	186	194	343	398	-	482	-	457	-	491	-	-	-
4	219	-	-	-	-	-	386	-	510	-	541	-	480	-	-	369	-
5	-	183	201	-	181	-	281	-	-	349	349	-	346	-	-	-	-
6	173	-	184	-	-	-	-	474	436	474	-	-	387	-	-	350	-
7	162	-	-	-	-	-	309	-	429	-	349	-	322	-	303	-	-
8	-	-	-	-	-	-	373	474	-	447	-	357	-	-	-	-	252
9	-	-	-	-	184	177	393	419	514	476	-	-	364	-	-	-	-
10	274	221	231	204	219	201	389	626	-	-	586	-	571	-	469	-	349
Mean	218	216	216	(244)	208	204	362	479	491	450	428	408					
S.D.	±51	±36	±33	±56	±28	±29	±48	±76	±68	±81	±89	±108					

* Times after commencement of operation

** B₁ = Day prior to operation

B₂ = Immediately prior to operation

post-operative levels. ($t = 0.0505$, $p = \text{N.S.}$). A slight fall of mean fibrinogen levels was detectable at the 120 minute stage, but this latter change did not reach significance levels ($t = 0.5428$, $p = \text{N.S.}$). All levels of fibrinogen elevation were significant in the days following operation (t values ranged between 5.1816 and 9.9711, $p < 0.001$), with maximum values being obtained on the third day post operation. The peak value was then followed by a slow decrease in concentration, with fibrinogen levels being still elevated on discharge from hospital.

(c) Fibrin Degradation Product Assay.

All results obtained are recorded in Table 5:11 and Fig. 5:10. It can be seen that there was a small increase in the circulating levels of F.D.P. in the operative period, although the change did not reach significance levels on the small number of results ($t = 1.9291$, $p = \text{N.S.}$). There was no significant difference between the two "before operation" samples ($r = 1.2742$, $p = \text{N.S.}$)

In the post-operative period, the changes were more marked. It can be seen that subjects H.1, H.2 and H.10, exhibited marked elevation of F.D.P. levels on either day 2 or 3 (Table 5:11 and Fig. 5:15). As these results were considerably at variance to the remainder of the assayed values (differing by over two standard deviations from the mean), they were excluded from calculation of the mean of the results. After calculating the means of the remaining results, there was now a significant increase, as compared with the pre-operative levels, of F.D.P., which persisted until the subject was discharged from hospital. (Table 5:12).

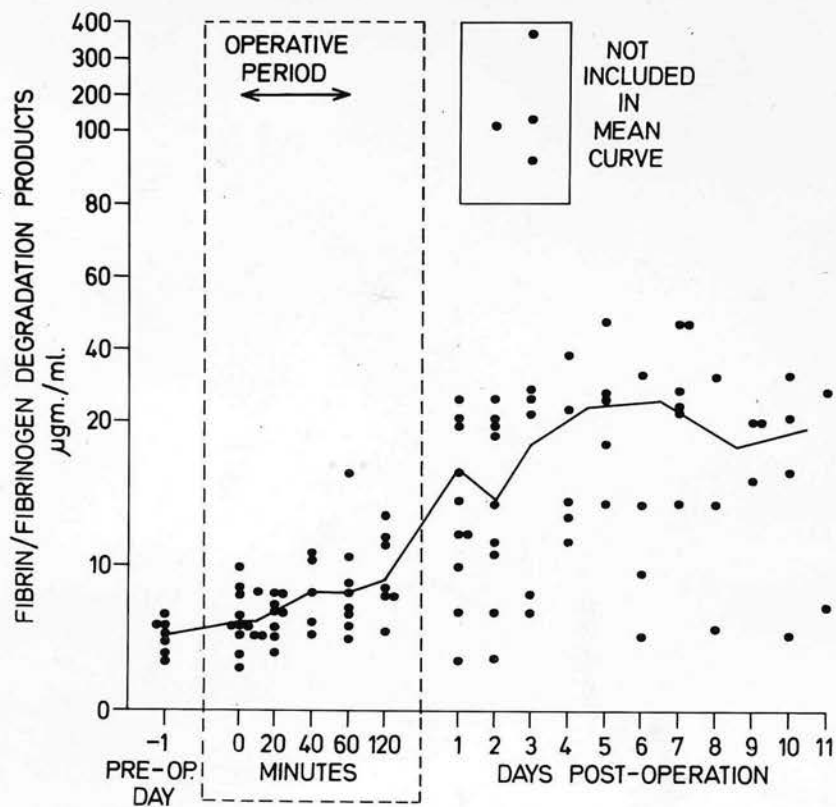


Fig. 5:10. Fibrin/Fibrinogen Degradation products prior to, during and following an intra-abdominal gynaecological operation.

TABLE 5:11.

FIBRIN DEGRADATION ($\mu\text{gm/ml.}$) PRIOR TO, DURING, AND FOLLOWING
AN INTRA-ABDOMINAL GYNAECOLOGICAL OPERATION IN ELEVEN SUBJECTS.

Subject	Pre-Operation		Operation Period - Minutes					Post Operation Period - Days										
	** B ₁	** B ₂	** 10	20	40	60	120	1	2	3	4	5	6	7	8	9	10	11
H 1	-	8.2	8.2	8.2	11.9	6.9	11.9	16.5	115.0*	132.1*	-	-	33.0	-	33.0	-	16.5	-
2	-	10.0	-	7.1	-	16.5	-	10.0	19.1	93.8*	-	28.3	-	-	-	-	-	-
3	5.0	5.9	-	5.9	-	5.9	8.1	23.0	23.1	-	23.1	-	9.6	-	14.4	-	-	-
4	3.6	7.2	5.4	5.4	10.8	9.0	5.4	7.2	-	21.6	-	14.4	-	14.4	-	-	21.4	-
5	-	6.5	-	4.1	-	5.0	-	13.4	11.0	8.1	13.5	18.5	-	22.5	-	20.1	-	-
6	-	3.0	-	-	-	8.3	5.9	6.9	6.9	6.9	38.1	-	-	47.4	-	-	33.0	-
7	5.9	5.9	-	7.4	5.9	-	-	14.4	11.8	-	11.8	-	5.2	-	5.9	-	5.2	-
8	5.4	5.4	5.4	7.2	5.4	7.2	5.4	-	3.6	-	14.4	-	14.4	-	-	-	-	7.2
9	3.8	3.8	-	-	-	-	-	14.4	14.4	28.8	-	-	-	28.8	-	-	-	-
10	5.9	5.9	-	8.3	8.3	8.3	11.8	19.9	19.9	366*	-	47.4	-	47.4	-	19.9	-	28.1
11	6.8	-	-	-	-	-	13.6	40.8	20.4	27.2	-	27.2	-	24.0	-	16.0	-	-
Mean	5.2	6.2	6.3	6.7	8.5	8.4	8.9	16.7	14.5	18.5	22.0		24.7		18.2		18.6	
S.D.	± 1.2	± 2.0	± 1.6	± 1.5	± 2.9	± 3.5	± 3.5	± 9.9	± 6.7	± 10.4	± 13.2		± 14.7		± 8.9		± 11.1	

* Not included in Mean.

** B₁ = Day prior to operation

" B₂ = Immediately prior to operation

** 10 = Times after commencement of operation

TABLE 5:12.

SUMMARY OF SIGNIFICANCE TESTS (PAIRED t TESTS) OF CHANGES
IN F.D.P. FOLLOWING SURGERY (GYNAECOLOGICAL OPERATION)

Pre-operation F.D.P.	:	Post-operation F.D.P.	Paired t Test	
Pre-operation	:	Day 1	3.2783	0.01>p>0.005
Pre-operation	:	Day 2	4.2871	0.005>p>0.001
Pre-operation	:	Day 3	2.8723	0.05>p>0.025
Pre-operation	:	Days 4 + 5	3.8532	0.005>p>0.001
Pre-operation	:	Days 6+ 7	3.8993	0.005>p>0.025
Pre-operation	:	Days 8 + 9	3.5004	0.02>p>0.01
Pre-operation	:	Days 10 + 11	2.5970	0.05>p>0.025

Patients H.1 and H.2 had uneventful post-operative courses; patient H.10 developed a clinically diagnosed deep venous thrombosis of the right leg and was subsequently treated with anti-coagulants.

B. Caesarian Section Operations.

(a) Euglobulin Lysis Activity.

The complete results are summarized in Table 5:13 and Fig. 5:11. The resulting levels of euglobulin lysis activity was very low as would be expected in late pregnancy. It was apparent that there was an

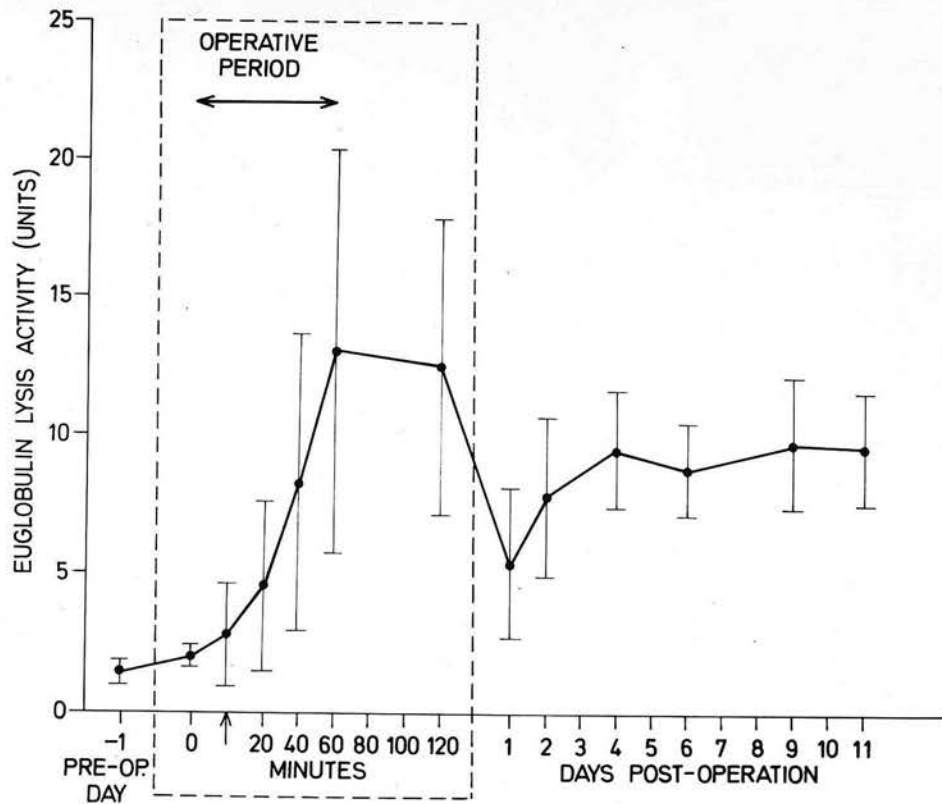


Fig. 5:11. Euglobulin lysis activity prior to, during and following a Caesarian Section operation.

TABLE 5:13.

FIBRINOLYSIS (EXPRESSED IN EUGLOBULIN LYSIS ACTIVITY UNITS) PRIOR TO,
DURING, AND FOLLOWING CAESARIAN SECTION IN FOURTEEN SUBJECTS

Subject	Pre-Operation		Operation Period - Minutes						Post Operation Period - Days										
	** B ₁	** B ₂	** CC	PR	20	40	60	120	1	2	3	4	5	6	7	8	9	10	11
CS 1	1.7	1.9	-	8.1	12.7	13.0	12.8	-	7.3	7.5	11.1	-	10.5	-	9.5	-	9.9	-	-
2	-	1.4	1.8	2.1	23.2*	48.0*	24.5	14.9	8.0	1.7	-	-	10.6	9.2	7.9	-	7.3	-	-
3	1.3	1.8	1.9	-	2.7	4.3	-	13.0	1.9	6.2	6.8	6.1	6.1	-	-	-	-	-	-
4	1.3	2.6	-	3.6	3.9	3.9	6.3	14.8	4.8	8.0	-	12.9	-	7.7	-	-	9.2	-	11.8
5	2.2	2.5	7.1	-	8.6	20.3	20.0	22.0	1.2	14.0	10.6	-	11.8	-	-	-	11.6	-	-
6	-	1.1	-	1.7	1.7	-	2.2	5.4	5.9	7.5	-	10.6	9.0	-	10.9	-	-	7.7	-
7	2.0	1.8	-	2.2	2.3	10.5	17.1	17.6	4.0	7.3	-	8.6	-	8.1	-	-	-	-	-
8	-	1.9	1.7	2.0	2.6	7.2	-	10.3	4.0	-	-	12.2	7.3	-	-	-	-	-	-
9	-	2.0	-	2.4	4.5	10.8	13.1	13.5	2.3	7.6	-	7.2	-	6.9	-	-	-	-	-
10	1.3	1.7	1.6	1.8	1.8	3.3	-	6.2	4.9	7.9	-	-	10.1	8.3	-	15.3	-	-	-
11	1.0	1.5	1.7	1.9	2.6	-	-	18.1	10.9	-	11.2	-	-	-	-	-	-	9.2	-
12	-	1.5	-	1.4	3.6	-	-	-	4.7	9.4	-	8.1	-	-	-	8.1	-	-	-
13	-	2.7	-	3.2	7.6	6.5	6.8	8.6	-	-	-	-	-	-	-	-	-	-	-
14	-	2.1	-	2.5	3.0	2.4	-	5.0	8.8	-	-	7.3	-	-	-	-	9.0	-	-
Mean	1.4	1.9	2.7		4.4	8.2	12.9	12.5	5.3	7.7	9.4		8.8		9.9		9.6		
S.D.	±0.4	±0.5	±1.9		±3.3	±5.5	±7.5	±5.4	±2.8	±3.0	±2.3		±1.7		±2.3		±2.1		

* Not included in Mean

** Abbreviations B₁ = One day prior to operation
B₂ = Immediately prior to operation

CC = Cord cut

PR = Placenta removed

20 = Minutes after PR, etc.

increase of lytic activity, reaching a maximum 60 minutes after the commencement of the operation and sustained until the 120 minute period. In the post-operative phase there was a depression of lysis activity in the first and second post-operative days, with a subsequent slow return to non-pregnant levels.

The increase in euglobulin lysis activity between the pre-operative day and that immediately prior to operation was not significant ($t = 1.6603$, $0.2 > p > 0.1$). There was also no significant difference between the pre-operative samples and the blood sample taken at the time of clamping of the umbilical cord and removal of the placenta ($t = 1.7068$, $0.2 > p > 0.1$).

However, within 20 minutes subsequent to removal of the placenta there was a significant increase in the euglobulin lysis activity ($t = 2.8882$, $0.01 > p > 0.005$) and this difference was sustained up until at least the 120 minute mark.

On the post-operative day, there was a significant increase of lytic activity over the pre-operative level ($t = 4.4146$, $p < 0.001$), and this was maintained until discharge. However, the lytic activity on the first two post-operative days was significantly less than that of the subsequent days.

(b) Fibrinogen Assays.

The results are summarised in Table 5:14 and Fig. 5:12.

The mean resting levels of fibrinogen was elevated as compared with the non-pregnant concentration. There was no difference between the two "before operation" samples ($t = 0.8722$, $p = \text{N.S.}$). There was again a slight fall in fibrinogen levels during the operative period, but the decrease was not significant ($t = 0.0705$, $p = \text{N.S.}$).

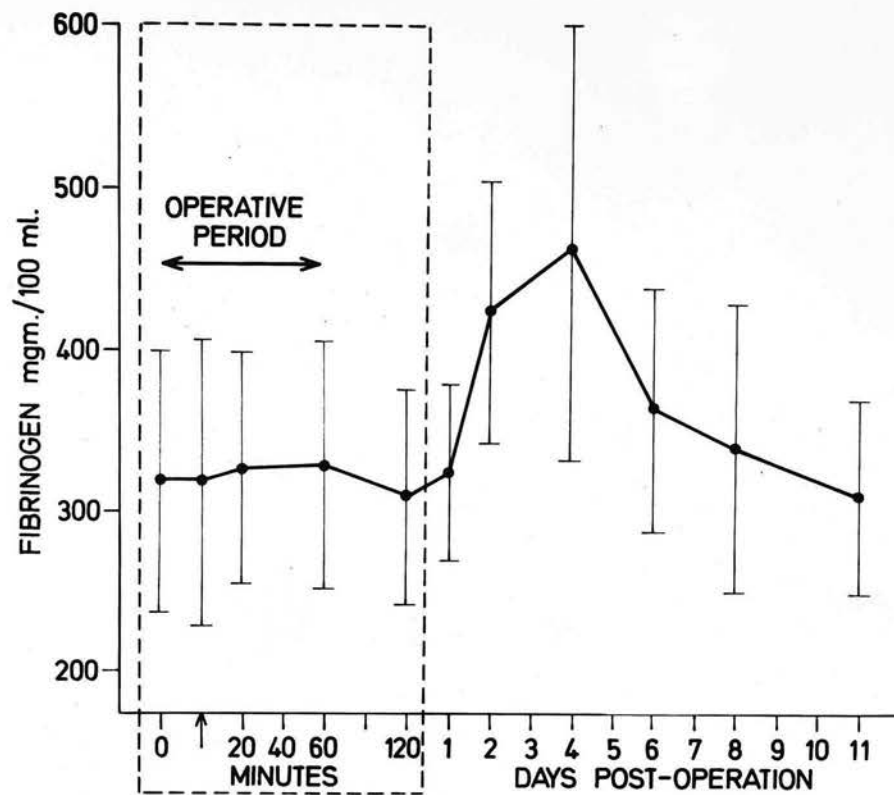


Fig. 5:12. Fibrinogen levels during and following a Caesarian Section operation.

TABLE 5:14.

FIBRINOGEN LEVELS (mgm/100 ml.) PRIOR TO, DURING, AND FOLLOWING
CAESARIAN SECTION IN ELEVEN SUBJECTS.

Subject	Pre-Operation		Operation Period - Minutes						Post Operation Period - Days										
	** B ₁	** B ₂	**CC	PR	20	40	60	120	1	2	3	4	5	6	7	8	9	10	11
CS 1	274	-	-	-	-	-	-	-	286	361	463	-	311	-	273	-	266	-	-
2	-	385	350	368	369	279	301	350	-	392	-	-	346	346	312	-	334	-	-
3	-	445	400	-	425	420	-	410	395	446	680	570	-	-	-	-	-	-	-
4	236	194	-	202	260	-	-	251	261	376	-	360	-	360	-	-	305	-	317
5	-	293	357	-	326	372	384	344	312	365	257	-	359	-	-	-	300	-	-
6	-	230	-	242	203	-	251	225	284	486	-	421	494	-	468	-	-	411	-
7	329	308	-	349	352	403	324	358	414	599	-	570	-	463	-	-	-	-	-
8	-	434	442	458	412	439	-	349	376	-	-	589	367	-	-	-	-	-	-
9	-	220	-	175	-	266	226	254	269	362	-	300	-	238	-	-	-	-	-
10	302	369	241	262	304	-	-	277	314	-	-	-	-	-	-	-	-	-	-
11	-	396	-	266	292	-	-	-	-	451	-	451	-	-	-	311	-	-	-
Mean	315		316		327	333		313	323	426	466		364		341		322		
S.D.	±81		±91		±71	±74		±63	±57	±79	±136		±76		±87		±49		

** Abbreviations - B₁ = One day prior to operation
B₂ = Immediately prior to operation

CC = Cord cut
PR = Placenta removed
20 = Minutes after PR, etc.

The increase of fibrinogen levels on the first post-operation day was not significant ($t = 0.2600$, $p = \text{N.S.}$) but changes in Days 2, 3 and 4 were highly significant when compared with pre-operative levels ($t = 3.2384$, $0.005 > p > 0.001$; $t = 3.3391$, $0.005 > p > 0.001$, respectively). The peak level of fibrinogen occurred on the fourth day post-operation although only a few blood samples were obtained on the third day post-operation. There was a subsequent continued fall of fibrinogen levels until discharge from hospital.

(c) F.D.P. Assay.

The complete results are summarized in Table 5:15 and Fig.5:13. The mean resting level of F.D.P. is at the upper level of the normal limits, this being consistent with the gestational period. An elevated value of F.D.P. on subject CS.4 was not included in the mean results.

The increase of F.D.P. throughout the operation was significant at the 5% level ($t = 2.3020$, $0.1 > p > 0.05$). Post-operatively, although a definite trend is apparent, similar to that of the other surgical groups, the levels of significance were not as great when compared with the pre-operative level. However, when compared with the normal non-pregnant level of F.D.P., the changes were then highly significant.

Patients CS.2 and CS.5 exhibited marked changes in F.D.P. levels in either the operative or post-operative phases. These changes followed a different pattern from the remaining twelve patients and the values were thus not included in the means. The results obtained in CS.2 and CS.5 are shown in Fig. 5:14, and referred to in the discussion.

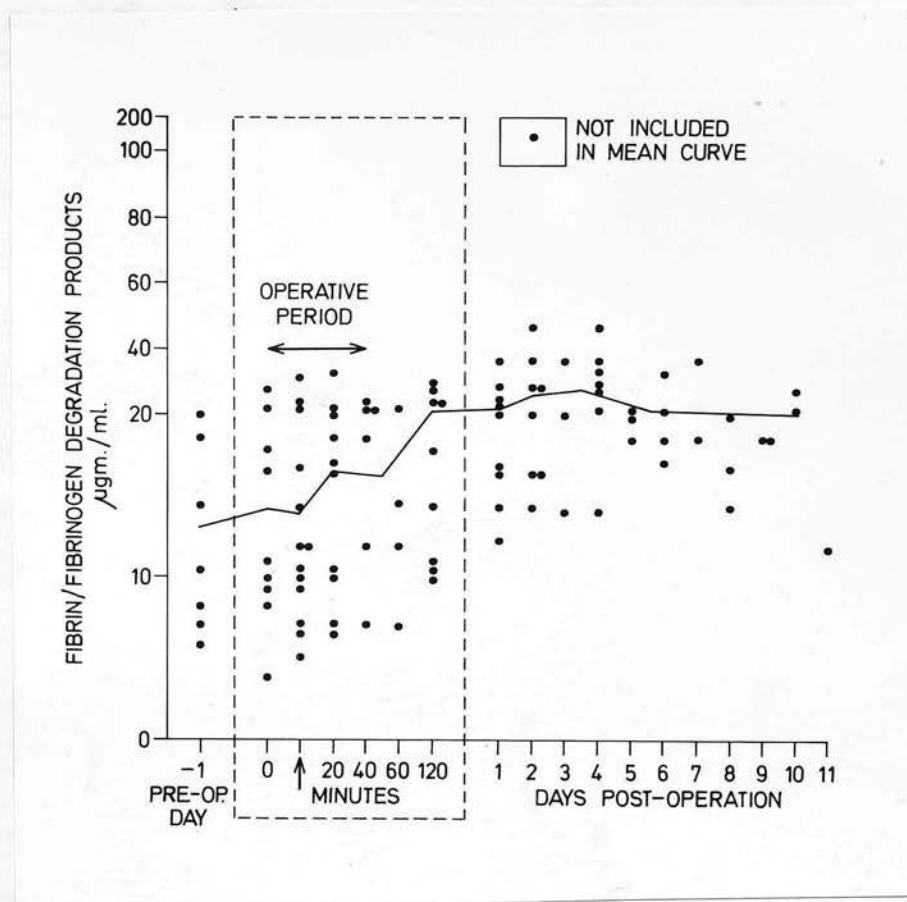


Fig. 5:13. Fibrin/Fibrinogen Degradation products prior to, during and following a Caesarian Section operation.

TABLE 5:15.

FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS ($\mu\text{gm/ml.}$) PRIOR TO, DURING, AND FOLLOWING CAESARIAN SECTION IN TWELVE SUBJECTS

Subject	Pre-Operation		Operation Period - Minutes						Post Operation Period - Days										
	** B ₁	** B ₂	** CC	PR	20	40	60	120	1	2	3	4	5	6	7	8	9	10	11
CS 1	18.6	9.3	-	9.3	18.6	18.6	-	-	37.1	37.1	37.1	-	18.6	-	18.6	-	18.6	-	-
2*	8.1	-	-	33.4	23.7	47.3	33.4	16.5	33.4	47.3	-	-	22.5	16.5	11.9	-	23.6	-	-
3	7.1	10.0	10.0	-	10.0	7.1	-	10.0	16.5	16.5	14.2	28.3	-	-	-	-	-	-	-
4*	5.9	8.3	-	11.9	16.5	11.9	11.9	23.7	23.7	149.0*	-	37.1	-	18.6	-	-	18.6	-	11.9
5*	14.0	14.9	-	38.9	62.1*	173.3*	110.7*	295.0*	90.1*	22.9	12.7	-	14.0	-	-	-	26.1	-	-
6	-	17.8	-	31.0	21.4	-	7.1	17.8	12.4	16.5	-	14.2	-	-	37.1	-	-	27.8	-
7	8.2	16.5	-	11.9	33.0	-	-	23.7	-	47.4	-	47.4	-	33.0	-	-	-	-	-
8	-	11.0	22.0	16.8	22.0	22.0	-	11.0	16.8	-	-	33.6	22.0	-	-	16.8	-	-	-
9	-	21.6	-	14.4	7.2	21.6	14.6	28.8	28.8	14.4	-	21.6	-	21.6	-	-	-	-	-
10	20.0	28.3	-	24.2	17.1	24.2	21.9	28.3	24.7	28.6	20.2	-	20.0	17.1	-	20.0	-	21.9	-
11	10.5	3.9	6.6	5.2	6.6	-	-	10.5	21.0	21.0	-	-	-	-	-	-	-	-	-
12	14.4	-	7.2	10.5	10.5	-	-	14.4	14.4	28.8	-	28.8	-	-	-	14.4	-	-	-
Mean	12.1	14.1	13.9	16.3	16.1	18.7	18.7	21.7	21.7	26.3	28.3	21.6	21.4	19.8					
S.D.	± 5.6	± 7.6	± 7.7	± 8.1	± 6.4	± 7.6	± 7.6	± 7.8	± 7.8	± 11.6	± 10.8	± 5.3	± 9.0	± 5.8					

* Not included in Mean.

** B₁ = Day prior to operationB₂ = Immediately prior to operation

CC = Cord cut

PR = Placenta removed

20 = 20 minutes after PR, etc.

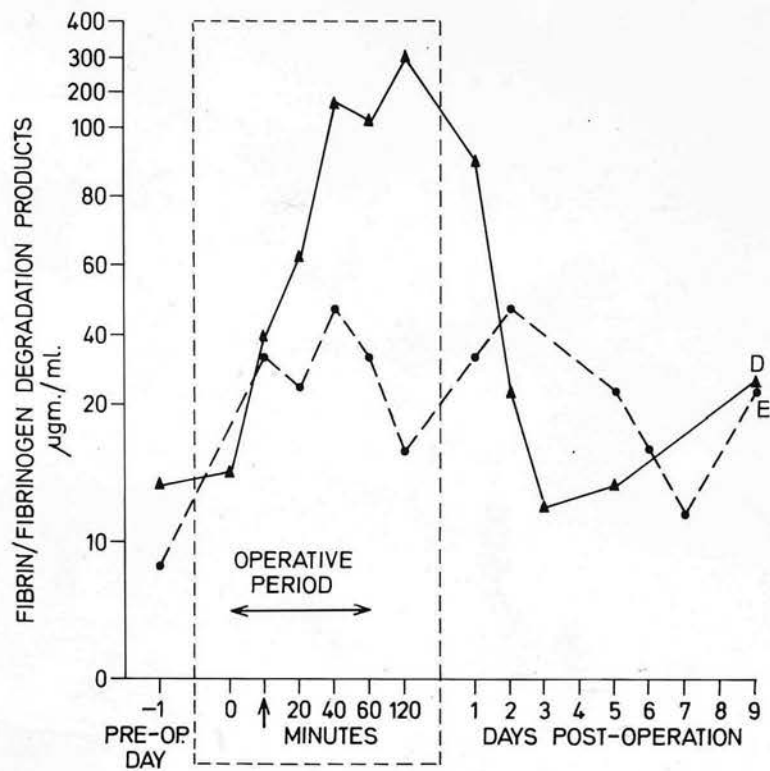


Fig. 5:14. Fibrin/Fibrinogen Degradation product levels in patients C.S.2 and C.S.5 during Caesarian Section operation.

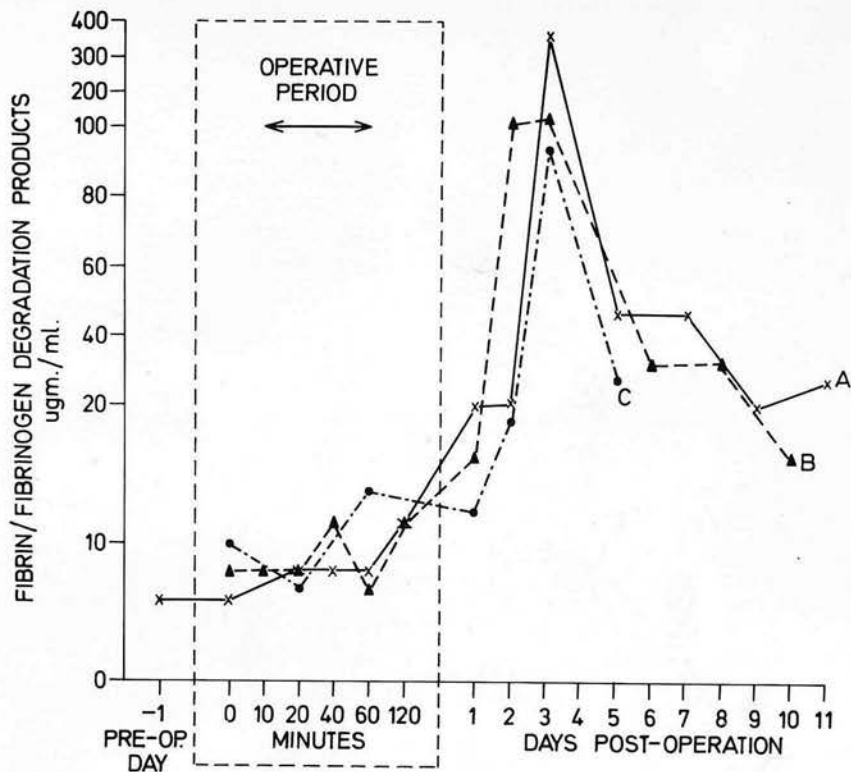


Fig. 5:15. Results of Fibrin/Fibrinogen Degradation Products Studies on subjects H.1 (B), H.2 (C), H.10 (A).

TABLE 5:16

SUMMARY OF SIGNIFICANCE TESTS (PAIRED t TESTS) OF CHANGES
IN F.D.P. FOLLOWING SURGERY (CAESARIAN SECTION)

F.D.P. (Caesarian Section)			
Pre-operation	: Post-operation	Paired t Test	
Pre-operation	: Day 1	2.2063	0.1 > p > 0.05
Pre-operation	: Day 2	2.2779	0.1 > p > 0.05
Pre-operation	: Days 3 + 4	2.9303	0.02 > p > 0.01
Pre-operation	: Days 5 + 6	2.1434	0.1 > p > 0.05
Pre-operation	: Days 7 + 8	1.3208	N.S.
Pre-operation	: Days 9 - 11	2.2701	0.05 > p > 0.025

C. Comparisons of Operative Results of Normal and Pregnant Subjects.

The response of the pregnant group to the operative stimulus, as measured in euglobulin lysis activity units, was less than in the non-pregnant subjects. The pattern of response was however similar, although the results suggest that lysis activity persisted longer in the pregnant group following operation. It could also be seen that the post-operative changes in the two groups, apart from the first day post-operation, are similar. (Fig.5:16.)

In Fig. 5:17 the results for the fibrinogen assays have been combined. The changes in fibrinogen levels follow the same pattern although the rise of

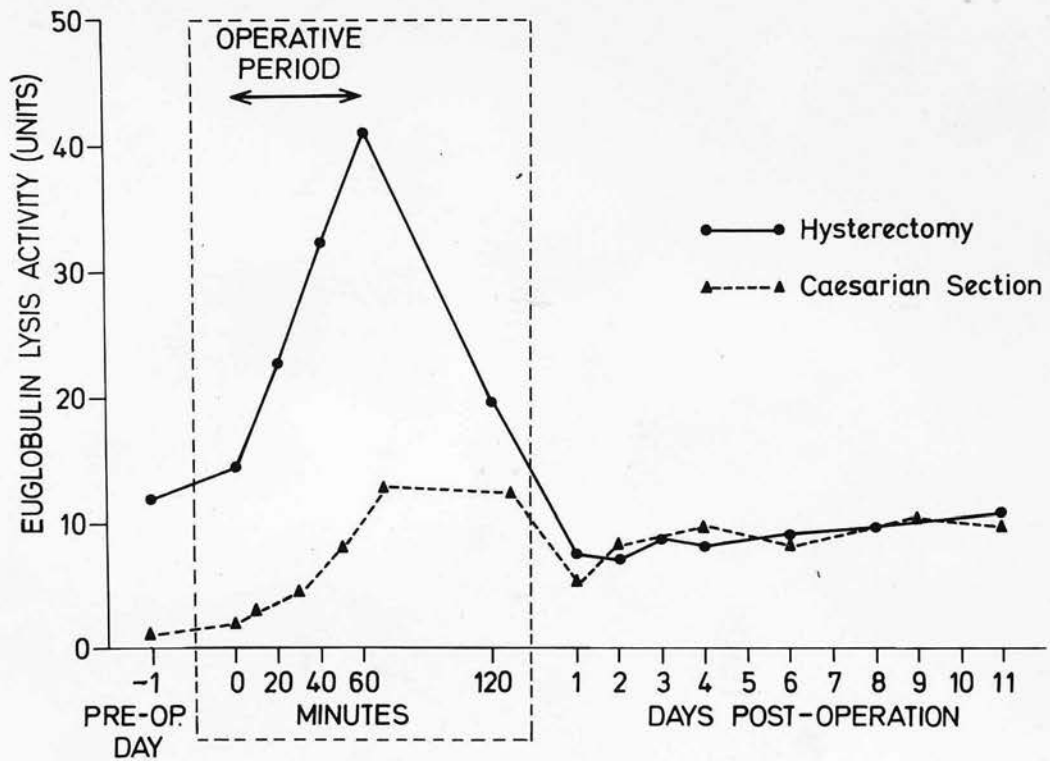


Fig. 5:16. Comparison of euglobulin lysis activities in gynaecological and Caesarian Section operation subjects.

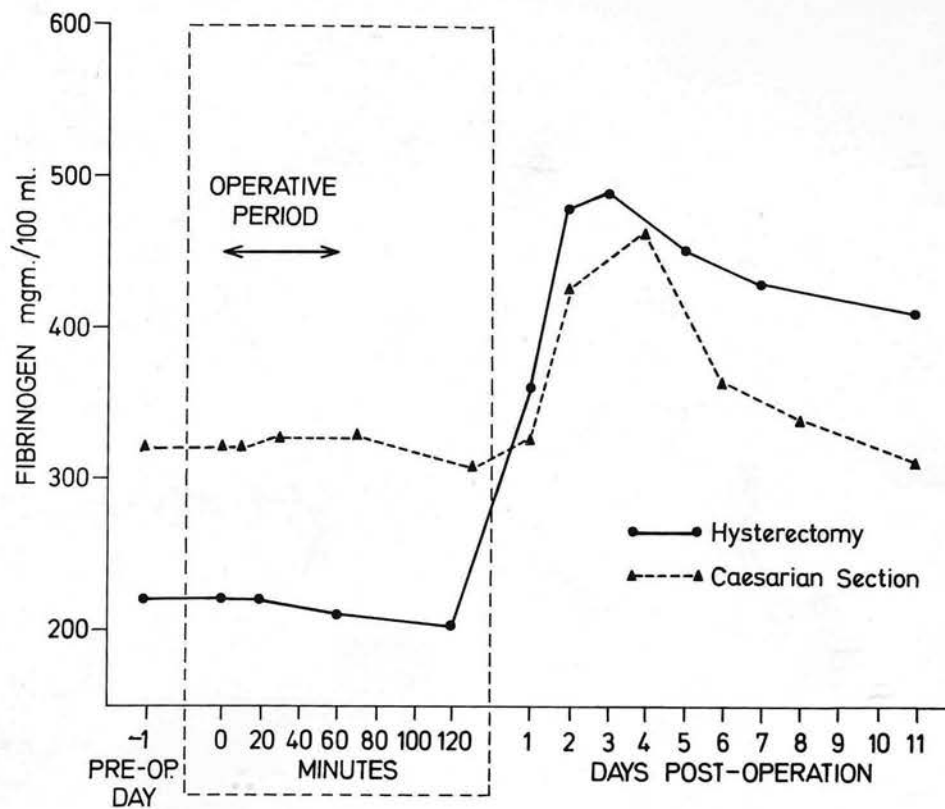


Fig. 5:17. Comparison of fibrinogen levels in gynaecological and Caesarian Section operation subjects.

fibrinogen was delayed to the second day in the pregnant group. Both groups reached similar mean values of fibrinogen, but the rate of fall in the pregnant group was, in these studies, more rapid than the non-pregnant group.

The change in F.D.P. in the operative period in both groups was small. In the post-operative period, the mean values in the Caesarian group are generally slightly higher than in the hysterectomy group but similar patterns are evident, with significantly elevated levels of F.D.P. persisting until discharge from hospital.

Apart from the euglobulin lysis activity, other parameters measured had not returned to non-pregnant levels by the time the patients were discharged from hospital.

DISCUSSION.

A. Intra-operation Changes.

(a) Euglobulin Lysis Activity.

Most investigators who have studied fibrinolysis during surgical procedures have used methods designed to measure circulating plasminogen activators (Franz et al., 1961; Andersson et al., 1962; Anderson and Mendelow, 1964; Olow, 1963). From these studies it has become clear that during the operative period, there is a variable increase in the level of lytic activity, which can be inhibited by the use of exogenous fibrinolytic inhibitors such as E.A.C.A. The exact mechanism by which plasminogen activator levels are increased is not known but factors such as pre-operative stress (McFarlane and Biggs, 1946), release of tissue activators into the circulation (Hardawey, 1966), and ischaemia (Kwaan and McFadzean, 1956) have all been regarded as providing evoking stimuli. Because of the numerous

factors known to increase activator levels, and also the lability, short half life and rapid changes that are known to affect the levels of this enzyme, it has not been possible to define adequately the exact effect of specific surgical procedures on fibrinolysis, as the changes occurring are undoubtedly complex. For these reasons Douglas et al (1966) believe that measurement of activator levels may yield misleading information and prefer to rely for evidence of activation of fibrinolysis on sequential plasminogen assays. Such an approach would appear entirely logical and using this technique they have been able to demonstrate that activation of fibrinolysis, as evidenced by lowered plasminogen levels does occur frequently during cardiac by-pass operations.

It is possible, however, that the increase of plasminogen activator levels during surgical procedures may be a response to the inevitable activation of blood coagulation that is assumed must occur during surgery to maintain haemostasis. If plasminogen activator plays an important part in the control of physiological fibrinolysis (Sherry et al., 1959) it might be expected that the intra-operative increase of plasminogen activator is a normal response of the body to maintain intravascular patency in the face of an increased local or systemic tendency towards fibrin formation. Intravascular coagulation could possibly provoke intravascular fibrinolysis and the rise of plasminogen activator levels would be a natural concomitant of the process.

If this hypothesis was correct, it should also be possible to demonstrate increased levels of the end products of fibrin or fibrinogen in the circulation during surgical operations representing in vivo activation of the fibrinolytic system.

In the present study, both changes in euglobulin lysis activity (representing both plasminogen activator and plasmin concentrations) and fibrin degradation products have been assessed in considerable detail, in an attempt to provide some answer to the relative importance of the alterations in euglobulin lytic activity occurring in association with intra-abdominal surgical operations in both normal and pregnant patients.

The studies have confirmed that euglobulin lytic activity increases steadily throughout the duration of surgery in both groups of patients. In terms of euglobulin lysis activity, the response was much less in the pregnant group but followed a similar pattern of increase, with maximum levels being attained approximately one hour from commencement of the operation. Levels in the non-pregnant subjects fell rapidly in the immediate post-operation period but appeared to be sustained in the pregnant group. It is difficult to compare the rate of increase of activator in the two groups, although from Fig. 5:16, it is possible that there was slower evolvment of activator in the pregnant group. Also, the peak of the activator increase may not have been attained in the Caesarian Section subjects as sampling was not performed between one and two hours post operation.

These marked changes in euglobulin lysis activity was complex in origin. Although it is conceivable that anaesthesia alohe may activate fibrinolysis, Bergentz and Nillson (1961) were unable to show an increase in euglobulin lysis activity after prolonged anaesthesia in dogs not submitted to any surgical trauma, and Cuocolo et al. (1965) could not demonstrate any systemic changes in fibrinolysis using a wide range of anaesthetic procedures. In the surgery groups described in this thesis, the length of time each patient

was anaesthetised before the surgical procedure commenced, was not noted in all cases; however, in six Caesarian Sections the time interval before the initial skin incision was 4.3 minutes, and in seven gynaecological operations, the interval was 12.6 minutes. In both instances, during this interval, no significant change in euglobulin lysis activity was noted; on the contrary, in some subjects the euglobulin lysis times, when studied at five minute intervals, tended to lengthen. Although the evidence is in no way conclusive it would appear unlikely that the administered general anaesthesia was significantly contributing to the observed intense activation of the fibrinolytic system.

Pre-operative stress of the patient, which was marked in some subjects was the most reasonable explanation of the slightly shortened euglobulin lysis times observed immediately prior to operation. However, comparison of the lysis times of the two "before operation" samples was not significantly different in either group and it is thus less likely that the shortening of lysis time during operation could be explained completely by endogenous stress, as suggested by Macfarlane and Biggs (1947).

The surgical procedure itself could have provoked the fibrinolytic reaction. With the incision and manipulation of various tissues, such as muscle and myometrial tissue, fibrinolytic tissue activators may have been released into the systemic circulation. Alternatively, as these tissues also contain thromboplastin, release of these agents may have promoted in vivo activation of coagulation, with a secondary response of the fibrinolytic system. In a small series of six closely studied patients, both pregnant and gynaecological, it was found that the first significant shortening of the euglobulin lysis time occurred 11.6 minutes (range 8-15) after the initial

skin incision, although the anaesthetic period prior to this time varied considerably - 8.8 minutes (range 1 - 15.5). This might suggest that the changes in fibrinolysis was related to tissue trauma although further study would be required to separate surgical effects from other possible factors. Such a study would not be easy as the many variables of surgery would be difficult to control.

With the Caesarian Section subjects, another factor may be relevant, Shaper et al. (1966) found that fibrinolysis in normal labours, as assessed by the euglobulin lysis time, increased immediately after delivery of the baby and before the cord was clamped. They attributed this increase of lytic activity to the stress of labour and also partly to a reduction of inhibitory mechanisms associated with removal of the placenta. During the Caesarian Sections, any effect of physical effort and subsequent adrenalin secretion of the mother is absent and it thus might be thought possible to assess the rapidity with which fibrinolysis returns to normal after placental removal. This initial hypothesis was, however, negated by the findings in the hysterectomy subjects, for it is apparent that the changes in lysis occurring with intra-abdominal operation alone will obscure the real pattern of change of fibrinolysis occurring following placenta removal. Notwithstanding, it will be seen from the results that at the time of clamping of the cord and removal of the placenta the euglobulin lysis activity was not statistically significant from the pre-operative level and it was not until 20 minutes after placental removal that fibrinolysis was significantly increased in a proportion of the subjects. By the 40 or 60 minute mark, however, all subjects were demonstrating increased lytic activity. Although it is difficult to separate the effects

of surgery from the effects of placental separation, it would appear that removal of the placenta did not result under these circumstances in a rapid marked increase of lytic activity. Again, it would be difficult to design an experiment to assess the effect of placental removal alone, in which the effect of extraneous maternal factors are excluded.

The increase of lytic activity appeared to be progressive throughout the operative period in the hysterectomy patients, and in some cases, lytic activity reached its maximum level in the blood specimen taken at the conclusion or subsequent to the operation. Although other investigators have investigated fibrinolysis within the operative period (Andersson et al., 1962; Olow, 1963) the timing of specimens has not always been mentioned. Anderson and Mendelow (1964) in a careful study of by-pass operation patients found that maximum lytic activity occurred between 30 and 75 minutes after the commencement of operation, and these findings are similar to those of Gans and Lowman (1967) who also studied by-pass patients. It is therefore possible to suggest that the changes occurring in lytic activity in the intra-abdominal operative situation are basically similar in timing, if not in degree, to those occurring in by-pass operations, and may represent some form of normal physiological response to a surgical stress. This would also be consistent with the findings in the Caesarian Section patients, although other factors may be influencing the degree of response. Also, it is possible to conclude that the peak lytic activity may occur subsequent to completion of the surgical procedure, as occurred in several of the gynaecological and Caesarian Section operations. Such a phenomena has been noted in cardiac by-pass operations (Douglas et al., 1966).

The mean fibrinolytic response to the operative stress in the Caesarian Section patients was much less than in the hysterectomy subjects. The pattern of response of the euglobulin lysis activity varied both in degree and time of onset between subjects. Although this may reflect to some extent variations in operative procedure and technique, it is now known that differences do exist in fibrinolytic (plasminogen activator) reactivity to a wide variety of stimuli (Cash and Allan, 1967), and that the fibrinolytic response is reduced, at least to an exercise stimulus, in late pregnancy (Woodfield et al., 1968).

Amongst the Caesarian Section patients, there were two subjects (CS.2 and CS.5) during the surgical procedure who exhibited relatively marked increases of euglobulin lysis activity. Both these patients had intra-operative complications and will be discussed further in the section dealing with F.D.P.

(b) F.D.Ps.

The presence of F.D.Ps. in serum is, in the present state of knowledge, incontrovertible evidence for either active primary or secondary fibrinolysis. As indicated in the introduction to this chapter, it might be expected that the lysis of fibrin, either as a response to increased fibrin formation or as a reaction to the release of tissue plasminogen activators, would be increased during surgical procedures. Evidence for this could be sited from the considerable increase of euglobulin lysis activity that occurs regularly during surgery. However, in these present investigations it can be seen clearly that the lysis of fibrin, as assessed by the assay of F.D.P., is increased only marginally during the operative period in both the obstetrical

and gynaecological patients, despite greatly increased levels of plasminogen activator.

The operative findings would support the supposition that immediate appreciable lysis of fibrin or fibrinogen does not usually occur as a direct response to surgical interference. It is quite possible, however, that other factors may activate fibrinolysis to an excessive degree with the production of elevated levels of F.D.P. The results in two of the Caesarian Section patients (CS.2 and CS.5) are illustrated in Fig. 5:14, and in these subjects it is apparent that F.D.P. has increased considerably during the operative period. Although the exact explanations of these findings are uncertain it is of interest to note that in one patient (CS.5) a large incision was inadvertently made in the placenta before access was obtained to the baby. The placenta was subsequently removed in several parts. No other exceptional circumstances were otherwise evident during the operation, but the sudden increase of F.D.P. at the time of clamping of the cord and placental removal might be regarded as circumstantial evidence of a relationship with the physical damage of the placenta. Euglobulin lysis activity in this patient was also well above the mean value of the other subjects although no significant changes in fibrinogen levels were recorded. As the placenta does not contain plasminogen activators (Albrechsten, 1957), but is rich in tissue thromboplastins, it is possible that either placental tissue or amniotic fluid was able to gain access to the maternal circulation with activation of coagulation and subsequent secondary fibrinolysis. Leary and Hertig (1950) have demonstrated that amniotic fluid may enter the maternal circulation via blood vessels opened by the uterine incision or perhaps via

the intervillous spaces if the placenta is incised. Amniotic fluid embolism has been reported during Caesarian Section operations (Leary and Hertig, 1950; Ellis and Nunan, 1956; Barno and Freeman, 1959; Scott, 1963; Willocks et al., 1966) and it would thus seem possible that in this patient a similar mechanism might have been operative, although not of sufficient degree to produce overt clinical symptoms.

The operation on the other patient (CS.2) was complicated by the presence of many intra-abdominal adhesions, and one pint of blood was transfused during surgery, followed by a further two pints post operatively. Although full details of operative difficulties are not now available, it appears probable that blood loss was excessive. Euglobulin lysis activity was greatly increased (Table 5:13).

In both these patients extraneous factors had complicated the normal pattern of fibrinolytic response to the surgery, resulting in the appearance in the circulation of excess lytic products, apparently, at least in one patient, without untoward clinical effects. These unexpected observations could be of some importance in the interpretation of the bleeding complications accompanying major surgery, for it is possible that the formation of excess levels of fibrin degradation product in other similar circumstances may herald the onset of a bleeding diathesis.

Generalised oozing from small blood vessels may occur during surgery and although this is usually of short lived or minor duration, on occasions it may be excessive, prolonged and intractable (Zucker et al., 1957). Both the bleeding time and platelet adhesiveness functions may be altered in fibrinolytic conditions associated with elevated levels of F.D.Ps. (Coopland

et al. 1968). The incidence of surgical oozing, particularly after major surgery, is significantly correlated with the occurrence of multiple changes in haemostatic parameters (Zucker et al., 1967) perhaps as a result of intravascular coagulation from the release of tissue thromboplastin into the blood (Hardaway, 1966) and consequent formation of F.D.P. It is therefore conceivable that "surgical ooze" may be in some way related to the changes occurring in F.D.P. and the effect of these polypeptides on blood vessels and platelets. Much more prospective investigation is required to evaluate this hypothesis.

B. Post Operation Changes.

(a) Euglobulin Lysis Activity.

The post-operative changes in euglobulin lysis activity are of considerable interest. Not only was there a marked variation in the pattern of response but differences exist between the two surgical groups.

In the hysterectomy group, there was a statistically significant decrease of euglobulin lysis activity after surgery. The explanation of this finding is not known, although Olow (1963); Bennet et al. (1967); and Littlewood (1967) were able to detect an increase of serum or plasma urokinase inhibitors post operatively in patients submitted to cholecystectomy, gastrectomy or hysterectomy operations respectively. The increases generally reflected the apparent decrease of lytic activity. No changes in antiplasmin activity was found. Although it is tempting to suggest that the change in urokinase inhibitors is the explanation of the decreased lytic activity, it has not yet been proved that the measurement of urokinase inhibitors is relevant to circulating plasminogen activator as urokinase cannot normally be demonstrated

in the peripheral blood (Kucinski et al., 1968). Measurements of specific plasma plasminogen activator inhibitors are required, but accurate assay techniques are not yet readily available. On the other hand, the decrease in activator levels may be due to increased consumption of this enzyme for fibrin lysis without rapid and adequate replacement. The finding of increased F.D.P. levels post-operatively might support this supposition but definite proof is lacking.

In the Caesarian Section group, the pattern of post surgical euglobulin lysis activity followed closely that of the gynaecological group, apart from the first two days post-operatively when lytic activity was even further decreased. Shaper et al. (1968) have demonstrated that plasma urokinase inhibitors fall slowly following normal delivery, and Nilsson and Kullander (1967) state that anti-activator activity also decreases gradually after delivery. This early post-operative decrease of euglobulin lysis activity might therefore be explainable on the effect of remaining, but gradually disappearing, exogenous inhibitors of pregnancy coupled with the normal increase of post surgical fibrinolytic inhibitors. Alternatively, the post-operative decrease may represent the movements of a compensatory biological system which "overswings" following prolonged suppression throughout pregnancy.

The relationship of these changes in lytic activity to the incidence of thrombo embolism is not known. Certainly, the only known patient in this series to develop a clinically diagnosed deep venous thrombosis (H.10) exhibited minimal post-operative changes in lytic activity. It is still possible, however, that decreased lytic potential in the post-operative phase

could predispose towards venous thrombosis. Experiments in rats and rabbits have shown that after E.A.C.A. administration and femoral vein ligation, the incidence of thrombi is increased over that of control animals (Borgstrom et al., 1969; Bergentz and Nilsson, 1961). Much further work is required as the predisposition towards post-operative venous thrombotic phenomena is almost certainly multifactorial.

(b) F.D.P.s.

Of no less interest are the alterations in levels of circulating F.D.P. occurring on the post-operative days. The significant elevation of F.D.P. in both groups might be explainable on the repair processes that follow the surgically induced damage to tissue. Extravascular tissue repair processes with deposition and resolution by lysis of locally deposited fibrin, could result in increase of intravascular levels of F.D.P. although there is no available evidence as yet to support this concept. Plasminogen activator, generated at the time of surgery, may actively be adsorbed onto fibrin as it is being laid down in tissues and subsequent slow local lysis might result in a continuous and steady release of lytic products in the post-operative period.

An alternative explanation of the increase is that the release of products of coagulation, such as tissue thromboplastin and serum, from damaged tissue into the circulation promotes a low grade intravascular coagulation syndrome with secondary fibrinolysis. Such a hypothesis might be investigated by observing the effects of heparin in a group of surgically treated patients on the post operation increase of F.D.P. as it might be expected that if the coagulation rate is decreased F.D.P. formation would also be reduced. On the other hand, tissue activators of plasminogen may be released from traumatised

tissue although this is a less likely explanation as the euglobulin lysis activity, would, if this were the situation, be increased rather than decreased in the post-operative period. It is also possible that small episodes of intravascular fibrin formation may commonly occur after surgery and the F.D.P. increase is a reflection of a compensatory fibrinolytic activity.

The post-operative changes of the gynaecological and obstetrical patients were similar, although the initial levels of pre-operative F.D.P. in the pregnant groups were higher as would be expected from patients studied late in the third trimester. In the first two post-operative days, the F.D.P. concentration is slightly higher in the Caesarian Section patients than in the gynaecological patients, but subsequently the levels are similar. As the Caesarian Section operation, in most instances, is associated with greater tissue trauma than the hysterectomy operation, it might be expected that post-operative F.D.P. values would also be higher if F.D.P. values are related in some way to the degree of tissue damage. That this was not clearly so could be considered as evidence against a simple release of F.D.P. from regenerating tissue; on the other hand, it may be that the physiological control mechanisms for the control and excretion of F.D.P. levels are similar in both groups of patients.

It is quite clear that until more is known of the basic physiological control of coagulation and fibrinolysis as well as the anatomical pathways and biochemical routes of excretion of F.D.Ps, further progress in our understanding of these dynamic changes may be delayed.

Four patients exhibited a marked increase of F.D.P. either on the second or third day post operation (CS.4, H.1, H.2 and H.10). The

first three subjects made an uneventful recovery from their surgery; the fourth subject developed a clinically diagnosed deep venous thrombosis of the right leg, and was commenced on anticoagulants.

The evidence for the deep vein thrombosis was not entirely conclusive. This patient (see appendix) had, on a previous post surgical occasion, developed a deep vein thrombosis, and had had intermittent leg pain subsequently, usually associated with her periods. On this admission, and three days after surgery, sudden severe pain developed in the same leg, and this was accompanied by an increase in calf diameter of $\frac{1}{2}$ " to $\frac{3}{4}$ ". There was local tenderness but Homan's sign was negative. In view of the past history she was immediately commenced on anticoagulants and, apart from other minor surgical complications, made an uneventful recovery. No further swelling of the leg occurred. Radiological investigations were not undertaken.

The diagnosis cannot therefore be established conclusively although the circumstantial evidence was strong. The finding of a high level of F.D.P. within three hours of the first clinical symptom is of considerable interest. F.D.P. have been noted previously in patients with embolic phenomena such as disseminated carcinoma with multiple thrombi and venous thrombosis (Merskey et al., 1966). These authors comment that the amount of lytic products found appeared to be correlated with the amount of intravascular fibrin formed. It is difficult to conceive alternative reasons for the elevation of F.D.P. in patient CS.10. The F.D.P. concentration on the previous day was normal and it must be assumed that the fibrinolytic response was secondary to the formation of either a localised or disseminated episode of intravascular coagulation.

The increase of F.D.P. may not necessarily have been a direct result of the formation of a deep vein thrombosis although this is one possibility. When clotting takes place, thrombin is adsorbed into fibrin (Quick, 1957). With contraction of the clot, serum rich in thrombin may become separated and, if this is released into the general circulation, may evoke an episode of disseminated intravascular coagulation with formation of lytic products of fibrin. Such a mechanism has been suggested for syndromes of intravascular clotting occurring in relation to pulmonary embolism (McKay et al., 1967).

Only a small fraction of patients with thrombo embolic phenomena exhibit clinical symptoms (Sevitt, 1965; Wessler, 1965), and perhaps only 50% of cases of pulmonary embolism are diagnosed before death (Smith et al., 1965). Surgery is well known to be associated with an appreciable incidence of post-operative thrombotic complications (Sharnoff et al., 1963; Storm, 1967), and in a series of post mortem studies on post-operative patients, the peak incidence of pulmonary embolism was found in the first post-operative week (Morrell and Duni, 1968), these results being similar to those of Dehlinger and Riemenschneider (1949) and Evans and Boller (1947). It was also noted that 62.8% of the patients who had had an operation had definite evidence of pulmonary emboli, although these were not always the cause of death. In view of these known facts, the finding of exceptionally elevated levels of F.D.P. in the early post-operative period in patients with no overt clinical symptoms may be of some importance. Although it is not possible at this stage to provide definite evidence, it might be reasonable to suggest that the increases in F.D.P. could reflect

isolated episodes of intravascular fibrin formation with subsequent lytic activity. Further intensive studies are required, particularly if other methods of diagnosing the formation of venous thrombi (Reich et al., 1966) at an early stage can be utilized. Such investigations may support the more extensive use of anticoagulants following surgery in selected groups of patients, known to be at exceptional risk, as in prostatic surgery (Held, 1965), or even after general surgery (Sevitt and Gallagher, 1959).

(c) Fibrinogen.

Shaper et al. (1968a) comment on the lack in the literature of detailed information of fibrinogen levels following surgery. In their study of a total of 21 African women undergoing Caesarian Section, the plasma fibrinogen concentration increased progressively until the fourth post-operative day and thereafter remained significantly elevated until the seventh day. These results are very similar to those reported in this study.

The plasma fibrinogen level in this present investigation was not significantly different from the pre-operative level on the first post-operative day; this being in marked contrast to the rise on the first post-operative day in the hysterectomy patients. Godal and Skaga (1966) who treated five patients undergoing surgery for peptic ulcer with trasylol, a fibrinolytic inhibitor, found that the post-operative rise of fibrinogen was less steep than in control patients. In view of the significant depression of euglobulin lytic activity on the first and second post-operative day in the Caesarian Section patients, it is conceivable that the lack of increase in fibrinogen could be a reflection of endogenously available inhibitors still circulating post delivery, perhaps coupled with a post-

surgical increase of inhibitors as described by Olow (1963) and Bennet et al. (1967). Some support for this hypothesis is derived from the work of Shaper et al. (1968a) who demonstrated that the increased urokinase inhibitor levels of pregnancy fell gradually and progressively rather than precipitously following delivery.

The fibrinogen levels in the hysterectomy patients rose sharply post operatively, even though there was a significant post-operative decrease of euglobulinlysis activity. This inhibition of the post-operative days was not as marked as in the Caesarian Section group, and was less constant. Although Bennett et al. (1967) have been able to demonstrate a rise in urokinase inhibitor following surgical operations that mirrors the depression of euglobulin lysis activity, it is still possible that the fibrinolytic inhibitors present in the post Caesarian and post hysterectomy situations may be dissimilar in both type and/or action and/or concentration and have different effects on the response of fibrinogen to surgical stress. Further experiments to assess the effect of different exogenous inhibitors on the post-operative rise of fibrinogen would be of interest, to clarify the influence of fibrinolytic inhibitors on the metabolism of fibrinogen. A clarification of the importance of the fibrinolytic inhibitor system to in vivo thrombolysis would also be a major contribution to our understanding.

In other respects the fibrinogen changes post-hysterectomy follow the pattern as described by other workers, although direct comparisons are difficult. Peak levels of fibrinogen were found on the third day post operation by Warren et al. (1950); Godal and Fichera (1961); Olow (1963); and Bennett et al. (1966), and most investigators agree that at least three

weeks are required for the plasma fibrinogen to return to pre-operative levels. Apart from the present study and that of Shaper et al. (1968a) there are no other reported studies of post Caesarian Section fibrinogen levels available.

It was of interest that despite widely different baseline values of fibrinogen concentrations in the two studies, the peak elevations were similar. As these two groups of patients were similar in age and all were pre-menopausal, the similarity of responses suggests that there is a homeostatic mechanism involved in the control of fibrinogen levels. However, neither the mechanism nor the stimulus for the production of hyperfibrinogenaemia is known (Eisenberg, 1966), and increased levels occur in a variety of clinical conditions. These rises have usually been attributed to a nonspecific response to stress and fibrinogen has been included in the "acute phase reactive" group of proteins by Crockson et al. (1966). Clearly, there is considerable room for investigation. The speed of rise and fall of plasma fibrinogen could be related to its in vivo turnover but evidence is lacking. The role of the liver in fibrinogen metabolism as suggested by Foster and Whipple (1922), and Ham and Curtis (1938), has not been defined and the relationship of changes in fibrinogen to alterations in other proteins could also prove interesting.

The recent work of Kropatkin and Izak (1968) is also relevant as these workers appear to have demonstrated that some thrombin or urokinase proteolytic products of fibrinogen or fibrin may provide the stimulus for increased production of fibrinogen by the liver. This new concept might be regarded as consistent with the present findings of increased F.D.P. after surgery. The measurement of the thrombin proteolytic products of fibrinogen

("coagulation peptides"), following surgical procedures might also provide further evidence for the concept of intravascular coagulation in these situations.

The role of raised plasma fibrinogen levels in the pathogenesis of thrombotic disease is not known (Ogston and Ogston, 1966). Although fibrinogen levels have been reported to be elevated in patients with ischaemic heart disease (Merskey et al., 1960; Katz et al., 1963) not all reports are in agreement (Naimi et al., 1963). In a comparative study of African and European subjects (Franz et al., 1961) post-operative changes of fibrinogen concentrations in both groups were similar although the incidence of venous thrombosis was very much higher in the European group. These reports would appear to indicate that although hyperfibrinogenaemia may possibly be implicated in the pathogenesis of venous thrombosis, other factors are also operative. High levels of fibrinogen (above 1,000 mgm.%) can also be obtained in certain clinical conditions (e.g. replacement therapy in haemophiliacs) without apparent untoward effects. In the present study, the subject (H.10) who developed a deep vein thrombosis, did have the highest fibrinogen level of the hysterectomy group studied. It was also noticed that H.1, who had elevated levels of F.D.P. on Days 2 and 3 without clinical symptoms, also had high sustained levels of fibrinogen. Although these observations may be coincidental there is considerable room for further meticulous investigation, particularly if other parameters, apart from fibrinogen can be assessed simultaneously.

SUMMARY.

Euglobulin lysis activity, fibrinogen levels and F.D.P. concentrations

have been measured, in a linear study, prior to, during and following both Caesarian Section and intra-abdominal gynaecological operations. The main findings were :-

1. The fibrinolytic response to surgery, as assessed by the euglobulin lysis time assay, was decreased in the pregnant group. The post-operative changes in both groups were similar with slight but significant decrease of euglobulin lysis activity.
2. Fibrinogen levels in both groups followed a similar pattern, with marked post-operative increase.
3. F.D.P. levels were not increased significantly in the operative period despite high levels of euglobulin lytic activity. In both groups of subjects, F.D.P. values were elevated above the normal non-pregnant levels in the post-operative period.
4. One patient with a deep venous thrombosis exhibited very high levels of F.D.P. on the third day post operation; three other subjects had elevated levels on the second or third day post-operatively without clinical symptoms.

It is concluded that these new and interesting findings could provide a basis for further intensive investigation of the inter relationships of coagulation and fibrinolysis in the surgical patient.

CHAPTER IV.

HAEMOSTATIC PROBLEMS OF PREGNANCY.

Introduction.

In the previous studies outlined in this thesis, the variations of haemostatic parameters in response to exercise, adrenalin and surgical stress procedures have been considered. For these studies, coagulation and fibrinolytic assay techniques had been developed and standardised and the natural extension of this work was their application to clinical problems. In this section, therefore, potentially dangerous haemostatic problems occurring in pregnancy have been identified and extensively investigated, using as many specialised laboratory procedures as practical.

The studies are presented as a basis for the further discussion of the aetiology, diagnosis and treatment of the disordered haemostatic mechanism in pregnancy, and provide further confirmation of the concept of disseminated intravascular coagulation as an intermediary of certain generalised haemorrhagic syndromes. Although a proportion of these laboratory studies were performed retrospectively, it was impossible, owing to the close association between laboratory and obstetric staff to provide much of the information in the acute clinical phase. With the further development of such liaison it should be possible to provide an even more rapid and accurate diagnostic service thus directly effecting both maternal and foetal morbidity and mortality.

Methods.

The patients investigated in these studies were referred to the Coagulation Laboratory staff by Obstetricians of the Simpson Memorial Maternity Pavilion. Most patients were seen very soon after a coagulation abnormality was suspected. In some patients, it was possible to obtain serial blood specimens, and although it was not always practical to collect blood samples under ideal circumstances, the general principles for venepuncture, as outlined in the appendix, were observed.

After the initial urgent coagulation tests had been performed, residual plasma or serum samples were stored in small aliquots at -40°C . Relevant coagulation and fibrinolytic assays were then performed in a group at a later date. It was not always possible to obtain enough plasma or serum for complete research haemostatic investigations, and on occasions, technical considerations precluded complete studies. All patients, however, were tested for the presence of serum F.D.P.

The pilot investigations on patients with antepartum haemorrhage, retroplacental haemorrhage and intrauterine death were also performed in conjunction with the Obstetrical staff. As soon as possible after admission, blood was withdrawn for assay; the subsequent clinical histories were then assessed by personal observations coupled with reviews of the case notes.

The coagulation and fibrinolytic assays employed are documented in the tables included with each study.

1. ABRUPTIO PLACENTAE.

A relatively common problem presenting in most obstetric hospitals is that of the accidental concealed and/or revealed retroplacental haemorrhage associated with premature separation of the placenta (abruptio placentae). This syndrome can be characterised by multiple changes in the coagulation and fibrinolytic mechanisms, as well as by hypofibrinogenaemia. Two case studies are reported, representative of moderate and severe cases respectively.

Case Reports.

Patient A.

Mrs. H.N., aged 26, a paragravida 1 + 1, had been first pregnant in 1963. The pregnancy had been normal throughout the gestational period but at term she had developed a sudden rise in blood pressure to $170/100$ with foetal distress. A forceps delivery resulted in the birth of a live baby. A second pregnancy in 1967 was complicated by hypertension and ended in a complete abortion at 20 weeks. During the present pregnancy she had had mild hypertension and was followed closely at the ante-natal clinic, her blood pressure averaging $145/95$.

At the 23 week gestational stage, she developed very severe lower abdominal pain which became progressively worse, and was soon associated with vaginal bleeding. Foetal movements had not been felt since the previous day. On admission she was shocked, with a blood pressure of $100/60$ and a pulse rate of 60 per minute; the uterus was hard, tender and of a 26 week gestational size; vaginal bleeding was moderate. A syntocinon infusion was commenced and blood was matched. Coagulation investigations revealed a plasma

fibrinogen level of 85 mgm. per cent. Two bottles of blood were administered and six hours after admission, i.e. thirteen hours after the initial symptoms, she delivered, spontaneously, a stillborn baby, along with a small placenta adherent to which was a large amount of retroplacental clot (approximately 350 ml.). Bleeding was not excessive and the uterus was well contracted.

The puerperium was uneventful, but on the second day post partum she discharged herself from hospital, against medical advice. Serial laboratory results are tabulated in Table 5:17 and Fig. 5:18.

Patient B.

The obstetric flying squad was called to the home of Mrs. E.L., aged 33, after she had developed a severe revealed/concealed accidental haemorrhage. She was then at 21 weeks gestation and had been well during the pregnancy. There had been one previous child and during that pregnancy she had had mild toxæmia. There was no other medical history of note.

On initial examination her blood pressure was $140/90$, pulse rate 110 per minute, and the uterus was hard and tender, and equivalent in size to a 28-30 week pregnancy. Because of marked bleeding, she was immediately transfused with three bottles of O, Rhesus Negative blood as well as with Ringer's solution.

On arrival at the hospital her general state was very poor, as additional haemorrhage had occurred during transport. Vaginal examination revealed an undilated cervix, a presenting part could not be felt and it was impossible to perform a surgical induction. She was thus started on an intravenous syntocinon infusion. Her blood pressure at this stage was $90/60$

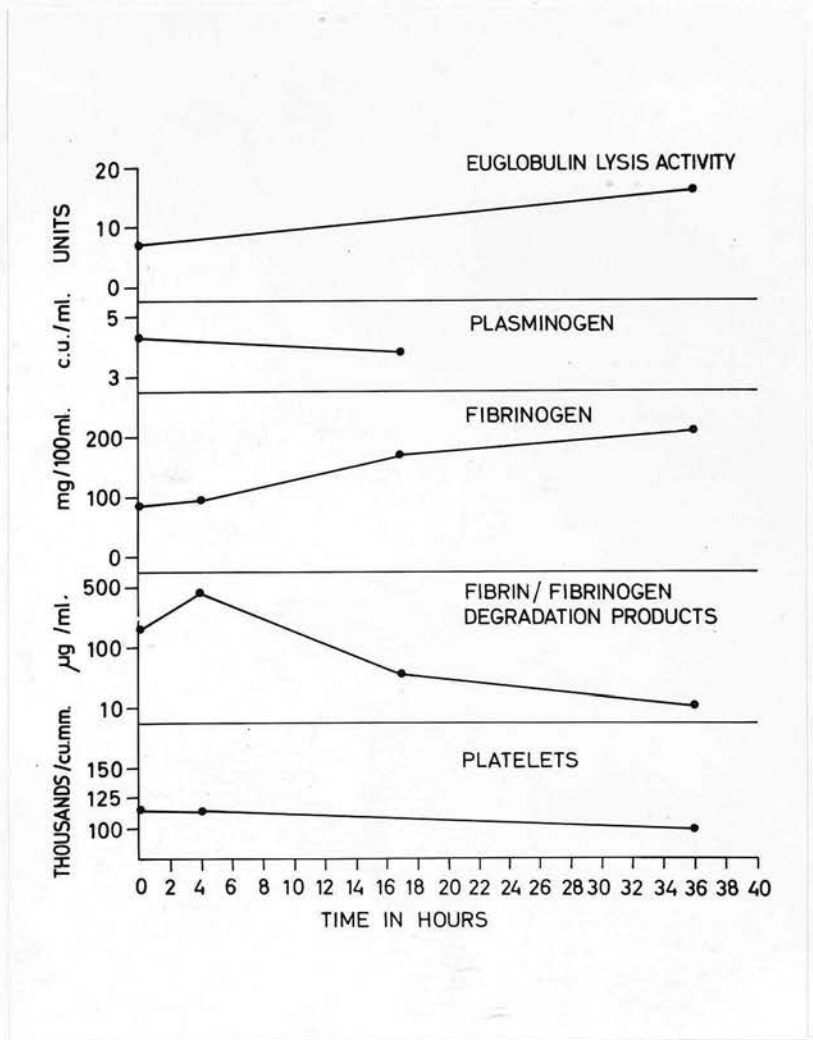


Fig. 5:18. Results of laboratory investigations on Mrs. H.N. (Abruptio placentae).

TABLE 5:17

RESULTS OF INVESTIGATIONS ON MRS. H.N. (MODERATE CONCEALED/REVEALED RETROPLACENTAL ANTEPARTUM HAEMORRHAGE)

Hours after Initial Symptoms	Euglobulin Lysis Activity (Units)	Fibrin Plates (mm ²)		Plasminogen c.u./ml.	Fibrinogen (mgm./100 ml.)		F.D.P. (μgms/ml.)	Platelets per cmm.	Haemoglobin (gm/100 ml.)
		Euglobulin	Plasma		Ellis Stransky	Thorpe Nephelometer			
9	7.0	784	144	4.3	85	117	230.0	115,000	10.4
13	-	-	-	-	95	89	461.0	115,000	11.0
26	-	-	-	3.85	170	141	37.6	-	-
45	15.9	400	100	-	212	194	14.4	100,000	11.0

and she was pale and shocked. An additional bottle of blood was administered and blood specimens were taken for future blood matching procedures. Bruising at venepuncture sites was excessive (Fig. 5:19).

Haemorrhage however continued and clotting investigations two hours after admission revealed incoagulable blood. The blood pressure was now $120/90$. Further blood was administered and she was sedated. Four hours after admission the blood pressure was $110/60$ and 8 gm. of Cohn Fraction I (clottable protein approximately 5 gm.) was infused along with two additional bottles of blood.

Six hours after admission her clinical condition was greatly improved. The blood pressure was now $120/60$, with a pulse rate of 120 per minute. A surgical induction was performed as haemorrhage was now much less. Eight-and-a-half hours after admission a stillborn foetus was delivered along with a large amount of retroplacental clot. She had been transfused a total of nine bottles of blood and 8 gm. of Cohn Fraction I, but her haemoglobin was still only 66%.

The puerperium was complicated by a B. Coli urinary tract infection which responded to Ampicillin. The low haemoglobin responded to iron therapy and a low platelet count also improved by the fifth day. She was discharged in good health and a post natal examination did not reveal any other abnormalities.

Serial laboratory results are tabulated in Table 5:18.

Comment.

The exact mechanisms by which the haemostatic changes occur in patients with abruptio placentae are not yet completely clear. Some



Fig. 5:19. Excessive bruising from venepuncture sites.
(Mrs. E.L., severe abruptio placentae)

TABLE 5:18

COAGULATION AND FIBRINOLYTIC RESULTS ON MRS. E.L. (SEVERE CONCEALED/REVEALED RETROPLACENTAL ANTEPARTUM HAEMORRHAGE)

Hours	Fibrinogen (mgms/100 ml.)		F.D.P. (mgms/ml.)	Platelets per cmm.	K.C.C.T. (secs.)			Prothrombin Time (secs.)	
	E.S.*	R.M.*			Test	Control	Mixture	Test	Control
0	55	-	1126.0	-	-	-	-	-	-
2	-	-	563.0	-	-	-	-	-	-
4	-	-	563.0	40,000	64	33	40	-	-
5 $\frac{1}{2}$	121	121	563.0	58,000	55	35	41	18	13
15	155	169	140.0	34,000	38	33.5	33.5	13.	11
33	227	222	26.0	16,000	-	-	-	-	-
57	253	244	35.0	56,000	-	-	-	-	-
81	222	198	8.8	-	-	-	-	-	-
132	322	348	17.6	325,000	-	-	-	-	-
180	272	-	6.6	-	-	-	-	-	-

* Abbreviations : E.S. = Ellis-Stransky Assay
R.M. = Ratnoff Menzies Assay.

authorities believe that the passage of tissue thromboplastin from placenta and decidua into the maternal circulation leads to a "consumption coagulopathy" in the plasma resulting in deficiencies of fibrinogen and several clotting factors (Schneider, 1959; McKay, 1965). Compensatory excessive fibrinolysis may further enhance this process with the production of either a- or hypofibrinogenaemia. In 10% of patients a severe bleeding syndrome results.

Alternatively, decrease of fibrinogen and clotting factors might be explained on local deposition of fibrin at the placental site (Pritchard and Wright, 1959; Willoughby, 1966). It has been calculated that loss of one litre of blood from the circulation will reduce the circulating fibrinogen pool by approximately 1.8 gm. and with spontaneous restoration of blood volume the plasma fibrinogen level will be reduced 50 mgm./100 ml. (Sherry, 1966). Pritchard and Wright (1959) claim that the hypofibrinogenaemia in abruptio placentae can be accounted for by the fibrinogen sequestered in the fibrin clot. Nilsen (1963) found that 2.5-20.0 gm. of fibrin could be recovered from the placental clot and this alone was regarded as enough to explain the hypofibrinogenaemia in 21 of their 24 patients. Although local deposition may be a factor in some cases of abruptio placentae it is difficult to explain severe hypofibrinogenaemia states solely on this mechanism.

In the patient under discussion it was more probable that a "consumption coagulopathy" or disseminated intravascular coagulation process was responsible for the hypofibrinogenaemia. The elevated levels of F.D.P. and lowered platelet levels indicated that intravascular coagulation had occurred with the formation of fibrin and secondary lytic activity. Specific coagulation factor assays were not performed but the finding of prolonged

clotting times in both the kaolin-cephalin and prothrombin time tests for Patient B is indicative of multiple decreases of clotting factors. This is confirmed by the satisfactory partial correction of these tests by the addition of normal plasma.

It is possible that D.I.C. occurs more frequently than previously suspected in patients with abruptio placentae. Basu (1967) found that in 20 cases of clinically suspected premature placental separation of which only six exhibited bleeding manifestations, high levels of F.D.P. were commonly found, often in association with normal levels of fibrinogen. In Table 5:19 can be seen the results of F.D.P. and fibrinogen assays in seven incompletely studied subjects with proven retroplacental haemorrhages. F.D.P. levels were elevated in most patients but not always to a degree commensurate with the volume of retroplacental clot. A possible explanation for the discrepancy could reside in the time at which blood specimens were removed. F.D.P. have been stated to have a half life of 9.6 hours (Fletcher et al., 1962), and if specimens are not taken in the acute clinical phase or soon after, the initial main F.D.P. rise may appear small or even not detectable. However, most of these specimens were removed soon after admission to hospital and usually within 4-6 hours of the first clinical symptoms. Alternatively, it could be concluded that only in a percentage of patients does thromboplastic material or serum gain entry to the maternal circulation, with subsequent activation of coagulation. However, Skjød t (1967a) in 20 patients with premature separation of the placenta was able to demonstrate the presence of "serum thrombotic accelerator" (Wessler, 1955) in each subject and this could be regarded as good evidence for the frequent occurrence of coagulation

activation in patients with abruptio placentae. Further studies with serial F.D.P. assays might clarify this problem.

Although the clinical picture in most patients with abruptio placentae is probably clear cut, there are many cases of ante-partum accidental hemorrhage in which the diagnosis is not known.

TABLE 5:19.

CLINICAL STATE AND LABORATORY INVESTIGATIONS IN SEVEN PATIENTS
WITH PROVED ABRUPTIO PLACENTAE.

Patient	Gestation (weeks)	Time lapse from initial symptoms to blood sample (hours)	Clinical Shock	Fibrinogen mgm/100 ml	F.D.P. μ gm/ml.	Volume Retroplacental clot (ccs)
1	32	2	Yes	-	230	400
2	32	10	Yes	224	57.6	"Large - $\frac{1}{3}$ of placenta"
3	37	$2\frac{1}{2}$ - 12	No	335	39.4	400
4	30	$\frac{1}{2}$	Yes	-	278	480
5	33	$6\frac{1}{2}$	No	160	63.0	600
6	36	?6	No	-	49.9	200
7	35	4	No	-	16.8	480

activation in patients with abruptio placentae. Further studies using serial F.D.P. assays might clarify this problem.

Although the clinical picture in most patients with abruptio placentae is reasonably clear cut, there are many cases of ante-partum accidental haemorrhage in which the aetiology of the haemorrhage is not known. A pilot study was therefore conducted to assess if the measurement of F.D.P. might aid in the detection of those patients with some degree of retroplacental haemorrhage as opposed to those with other ante-partum bleeding conditions. The results of 25 consecutive patients with ante-partum haemorrhages are recorded in Tables 5:19 and 5:20. It is apparent that in nearly all the patients with ante-partum haemorrhage, whether idiopathic or due to placenta praevia, there was no marked increase of F.D.P. This is in comparison with the seven patients with proved retroplacental haemorrhages in whom six a rise of F.D.P. was demonstrated. In one patient (16) (Table 5:20), in whom severe abdominal pain indicated the possible diagnosis of a concealed haemorrhage, the low level of F.D.P. was regarded as not being indicative of this diagnosis and subsequently a diagnosis of severe reflex oesophagitis was made. This small study indicates that, providing blood samples are obtained soon after the onset of symptoms, the measurement of F.D.P. may be of use in the diagnosis of abruptio placentae. Further intensive study of a wider range of subjects in greater detail is required to substantiate this hypothesis.

Numerous workers have attempted to find evidence of fibrinolytic activity in patients with abruptio placentae (Willoughby, 1966; Pritchard and Brekken, 1967) but the results have usually been equivocal. The explanation of this now appears clear. The techniques which have been

TABLE 5:20.

RESULTS OF F.D.P. ESTIMATIONS ON EIGHTEEN PATIENTS WITH ANTEPARTUM ACCIDENTAL HAEMORRHAGE.

Patient	Gestation (weeks)	Clinical Diagnosis	Final Diagnosis	Blood Loss	F.D.P. (μ gm./ml.)
1	33	A.P.H.**	Cervical polyp.	Slight	7.9
2	34	Placenta praevia	Placenta praevia	2 pint transfusion	23.9
3	31	A.P.H.	? Cause*	Slight	16.8
4	32	A.P.H.	? Cause*	Slight	15.8
5	37	A.P.H.	Small organised retroplacental clot	Slight but persistent	4.1
6	39	A.P.H.	? Cause*	Slight	4.0
7	38	A.P.H.	? Cause*	Slight	4.1
8	28+	A.P.H.	? Cause*	Slight	7.9
9	29+	A.P.H.	? Cause*	Slight	7.1
10	36	A.P.H.	? Cause*	Slight	12.7
11	36	A.P.H. Hypertension	Twins with one I.U.D.	Slight	16.8
12	37	A.P.H.	? Cause*	1 pint transfusion	2.9
13	35	A.P.H.	Anencephaly	Slight	6.4
14	33	Placenta praevia	Placenta praevia	Moderate	6.4
15	32	A.P.H.	? Cause*	Slight	4.1
16	36	Abdominal pain	Reflux Oesophagitus	Nil	3.0
17	37	A.P.H.	Placenta praevia	Slight	3.0
18	38	A.P.H.	Placenta praevia	Slight	6.4

* No retroplacental clot.

** A.P.H. = Accidental antepartum haemorrhage.

used have been dependent on clot lysis in in vitro systems and, unless special precautions are taken and the blood specimen is removed close to the time of release of plasminogen activator (or plasmin), the results may lie within the normal range. Plasmin is neutralized rapidly in vivo by antiplasmins and the half life of plasminogen activator is known to be 13 ± 5 minutes (Fletcher et al., 1964); thus the possibility of obtaining evidence of increased lytic activity is greatly decreased. The thrombin time, although sensitive to the presence of anti-thrombic agents, such as F.D.P., lacks both sensitivity and specificity, and cannot be regarded as an accurate test. The assay of F.D.P. as specific indicator of active in vivo fibrinolytic activity would therefore seem a more reasonable and valuable measurement. In both the present patients high levels of F.D.Ps. were noted and provided unequivocal evidence of excess lytic activity. The elevated levels of F.D.P. fell rapidly after institution of treatment and subsequent to uterine clearance.

Thrombocytopenia is common in severe D.I.C. and in Patient B, the changes were particularly marked. Platelet levels are often slow to increase after episodes of defibrination and McKay (1967) stated, that in his experience, this haemostatic parameter is the slowest to reach normal levels after a defibrination. It is of interest to note that Coopland et al (1968) were able to demonstrate decreased platelet adhesiveness and prolonged bleeding times in two cases of abruptio placentae carefully studied. It was suggested that such an abnormality of platelet and vascular function may be related to the known effect of F.D.P. on platelets (Kowalski et al., 1964). Further studies may well assist in assessing the importance of this observation.

There was no evidence of renal failure in these patients. Premature separation of the placenta is a common cause of renal cortical necrosis and has been stated to occur once in every 100-200 patients with this disorder (Williams, 1963). In the last five years in the Simpson Memorial Maternity Pavilion, of 362 patients with confirmed accidental revealed/concealed haemorrhages four patients developed renal failure.

McKay et al. (1953) have suggested that renal cortical necrosis following abruptio placentae is a direct consequence of a generalised Shwartzman-like reaction occurring in man. It has been established that bilateral cortical necrosis of the kidneys can occur in pregnant animals after only one injection of endotoxin (Apitz, 1935; Wong, 1962) and Margaretten et al. (1964) were able to produce renal cortical necrosis in pregnant rats after an intravenous infusion of thrombin. It is possible that the production of endogenous thrombin in man, as may occur in intra-vascular coagulation, may result in similar pathology, of varying degrees, developing. As decreased fibrinolysis in pregnancy has been implicated as a sensitising factor for the generalised Shwartzman reaction, the reactivity of the individual fibrinolytic system to a pathological stress, such as thrombin release, may be of some importance, perhaps determining whether complications such as renal cortical necrosis develop. In a previous section (5:2) it was shown that the ability of some pregnant women in the third trimester to respond to an exercise stress by generating plasminogen activator was reduced greatly and this finding could be in some way related to pathogenesis and incidence of renal cortical necrosis in pregnancy. However, some authorities (Sheehan and Moore, 1953; Sheehan, 1966) believe

that vascular spasm is responsible for the ischaemic necrosis and that thrombi form later as a consequence of ischaemic vascular damage. These approaches to the problem have stimulated a great deal of research endeavour, summarised adequately by Hjort and Rapaport (1965).

Both patients responded rapidly to the infusion of blood and/or a concentrate of fibrinogen. In Patient B the response to blood alone was less satisfactory. This is not altogether surprising in view of the fact that each bottle of blood contains only 0.75-1.0 gm. of fibrinogen, and will usually be deficient, to at least a moderate degree, of Factors V and VIII which are usually decreased in syndromes of D.I.C. These latter factors will only be available in fresh blood and their absence may contribute to the bleeding diathesis. However, the infusion of blood will supply a source of antiplasmins and this may aid in the reduction of excess plasmin activity. Verstraete et al. (1963) prefer to use heparin in association with fibrinogen infusion in an effort to reduce the risk of promoting additional intravascular clotting resulting from residual thromboplastic substances in the maternal plasma. Although this would appear a logical and reasonable therapy, Pritchard and Brekken (1967) point out that in their extensive study of 141 cases of severe abruptio placentae the only death occurred as a result of inadequate blood replacement and no evidence was found of vascular thrombotic obstructions. The importance of adequate uterine retraction in post partum haemostasis must also not be underestimated. (Hibbert and Jefcoate, 1966).

The use of proteolytic inhibitors in abruptio placentae is probably limited to the very uncommon case with severe generalised bleeding associated

with hypofibrinogenaemia, marked clot lysis and very rapid disappearance from the circulation of doses of fibrinogen usually regarded as adequate to raise the blood fibrinogen level. Even in such cases, the use of heparin to decrease the rate of coagulation may be more logical although the concomitant use of small doses of proteolytic inhibitor to reduce any residual lytic activity might not seem unreasonable. Amris and Kjeldsen (1966) reported the use of trasylol in abruptio placentae but the advantages (if any) of this therapy cannot be determined from the studies as both blood and fibrinogen were also used.

2. INTRA-UTERINE DEATH.

Another complication of pregnancy is intra-uterine death. The following one report describes haemostatic complications associated with the syndrome, but occurring subsequent to delivery.

Case Report.

Mrs. A.K., aged 29, was first seen at an out-patient clinic of the Simpson Memorial Maternity Pavilion in January, 1968. She had previously had two normal deliveries as well as a miscarriage at 10 weeks, but her medical history was otherwise uneventful.

She had again become pregnant and at the visit to the Clinic it was noted that the uterine size was small (16 weeks) for the calculated dates (20 weeks). Earlier the following month she was admitted to hospital for investigation of a brownish vaginal discharge. While in hospital slight vaginal bleeding became evident and the foetal heart could not be heard on the ultradrop. Evacuation of the uterus was therefore carried out and at

operation the uterus was found to be of 8-10 weeks size and contained a macerated foetus. There was minimal bleeding at this time.

Two hours later vaginal bleeding became profuse and coagulation investigations revealed low fibrinogen levels couples with other abnormal fibrinolytic tests.(Table 5:22). The blood pressure was $80/?$ and the pulse rate 100 per minute, and she was obviously shocked. An infusion of blood was commenced as well as 12.0 gm. Cohn Fraction I and 5 gm. of Epsilon Amino Caproic Acid (E.A.C.A.) Four hours after the initial bleeding her clinical condition was greatly improved and the vaginal bleeding settling. She was continued on a small dose of E.A.C.A. and her subsequent progress was uneventful.

The full details of all the coagulation and fibrinolytic studies are tabulated in Tables 5:21 and 22, and Figs. 5:20 and 21.

Comments.

Intra-uterine foetal death is not uncommonly associated with disorders of coagulation and fibrinolysis. Hodgkinson et al. (1964) state that hypofibrinogenaemia occurs in 25% of subjects when the duration of foetal death is five weeks or more. Lerner et al. (1967) thus suggest that fibrinogen levels should be measured at the time foetal death is diagnosed and thereafter weekly. If the level falls below 150 mgm./100 ml. the pregnancy should be terminated and similar policies are commonly followed in many obstetric hospitals in this country.

With this patient the diagnosis of intra-uterine death was not made prior to hospital admission. The history of recent but slight vaginal

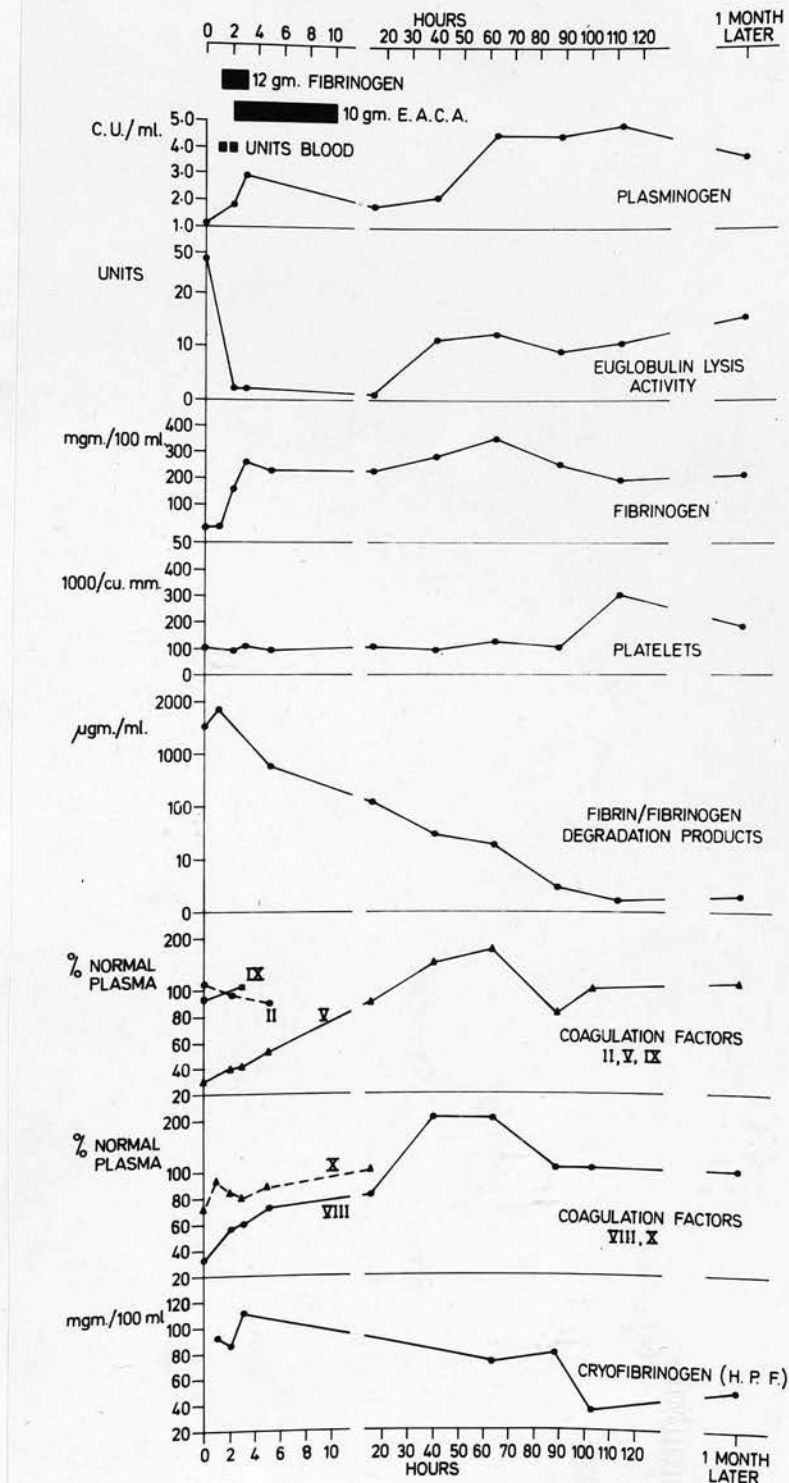


Fig. 5:20. Results of laboratory investigations on Mrs. A.K. (intra-uterine death).

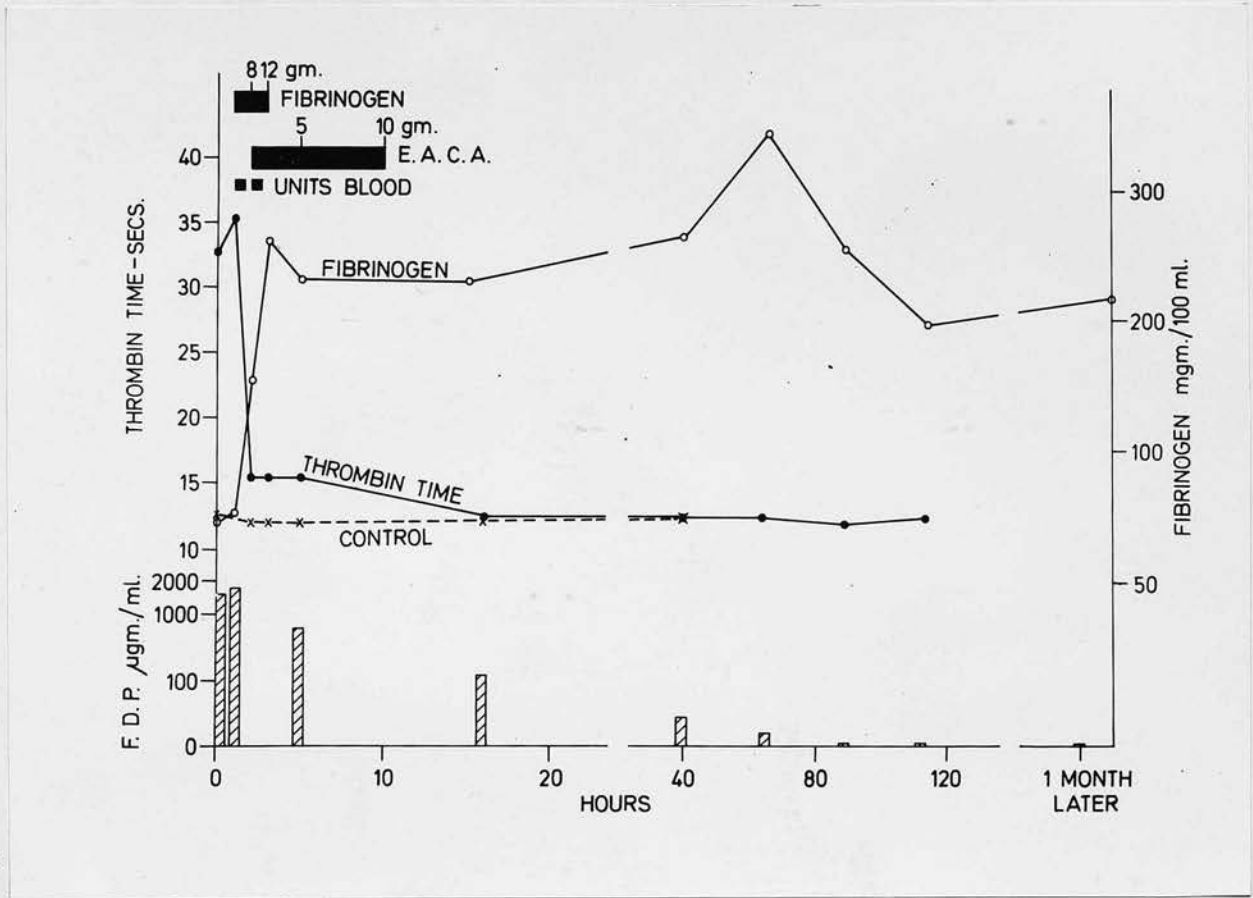


Fig. 5:21. Further laboratory results on Mrs. A.K. (intra-uterine death)

TABLE 5:21.

RESULTS OF COAGULATION AND FIBRINOLYTIC STUDIES ON MRS. A.K. (INTRA-UTERINE DEATH)

Hours after Initial Episode	Fibrinogen (mgm/100 ml.)			F.D.P. (μ gm/ml.)	Plasminogen (c.u./ml.)	Euglobulin Lysis Activity (Units)	Fibrin Platelets (mm ²)		H.P.F. (mgm/100 ml.)	Platelets per cmm.
	E-S*	T-N*	R-M*				Euglobulin	Plasma		
0	73	157	59	1,546.0	1.10	46.40	549	724	-	100,000
1	76	143	51	1,843.0	-	-	-	-	94.6	-
2	166	253	-	387.0	1.85	2.43	132	-	88.7	95,000
3	266	235	166	387.0	2.92	2.39	167	-	113.0	110,000
5	236	249	-	774.0	-	-	-	-	-	100,000
16	234	249	-	193.0	1.82	1.09	203	95	-	110,000
40	288	264	-	48.0	2.08	11.50	716	144	-	99,000
64	356	225	215	24.0	4.48	12.30	-	-	72.6	130,000
89	261	210	-	5.1	4.51	9.70	813	156	77.8	110,000
113	202	157	-	2.7	4.95	10.80	676	144	36.2	310,000
1 month later	222	197	-	3.0	3.70	15.66	-	307	51.8	185,000

* Abbreviations E-S = Ellis Stransky Assay
T-N = Thorpe Nephelometer Assay
R-M = Ratnoff-Menzies Assay

TABLE 5:22.

RESULTS OF COAGULATION AND FIBRINOLYTIC STUDIES ON MRS. A.K. (INTRA-UTERINE DEATH)

Time Hours	Coagulation Factor					Thrombin Time (seconds)			Arvin Time (seconds)		
	II (%)	V (%)	VIII (%)	IX (%)	X (%)	Test	Control	Mixture	Test	Control	Mixture
0	110	31	34	96	75	33.0	13.0	17.0	53.0	15.4	-
1	-	-	-	-	96	35.6	11.7	-	61.0	19.2	21.5
2	98	40	58	-	87	16.3	11.7	15.5	21.0	19.2	21.4
3	-	41	61	108	81	15.5	11.7	14.3	20.9	19.2	-
5	90	53	74	-	88	16.1	13.9	15.2	23.9	21.2	22.7
16	-	91	82	-	100	13.3	13.9	13.9	15.2	15.3	15.2
40	-	147	204	-	-	13.1	13.1	-	13.6	13.2	-
64	-	174	198	-	-	12.6	12.6	-	13.4	14.6	-
89	-	82	107	-	-	12.4	12.6	-	14.6	14.6	-
113	-	100	108	-	-	13.0	12.6	-	13.7	14.6	-
1 month later	-	109	103	-	-	-	-	-	-	-	-

haemorrhage coupled with the absence of foetal heart sounds was taken as evidence of a spontaneous miscarriage and the uterus was thus evacuated. No coagulation studies were performed prior to operation. In retrospect, it was apparent that the diagnosis was that of a missed abortion, and gauged by maternal dates and the size of the macerated foetus, death in utero had probably occurred 8-10 weeks earlier. It was thus quite possible that the initial vaginal haemorrhage may have been early evidence of a pre-existing coagulation defect which was aggravated by the operative procedure, as profuse bleeding did not commence until two hours after surgery.

The patient exhibited many of the clinical and laboratory hallmarks of disseminated intravascular coagulation. Clinically, the onset of haemorrhage associated with severe shock inconsistent with the degree of blood loss is common in the fully developed syndrome. The laboratory findings of lowered levels of coagulation factors V and VIII, a prolonged thrombin time, hypofibrinogenaemia, diminished levels of platelets, hypoplasminogenaemia, cryofibrinogenaemia and increased F.D.P. concentrations were all consistent with this diagnosis.

The euglobulin lysis time estimation was also greatly shortened and although this may have been a reflection of diminished fibrinogen and plasminogen levels in the euglobulin substrate, fibrin plate assays of plasma fibrinolytic activity also demonstrated increased lysis, this being attributed to either plasmin or plasminogen activator activity.

The linear study of both coagulation and fibrinolytic parameters subsequent to delivery are of considerable interest. It is apparent from Table 5:22 + Fig. 5:21 that F.D.P. are exerting an antithrombic effect on the

thrombin-fibrinogen reaction for at least 10-16 hours after the initial episode, by which time the F.D.P. levels had fallen to 193 μ gm/ml. Both thrombin and Arvin times were initially affected by lowered fibrinogen levels but the combination of prolonged thrombin time in conjunction with a normal Arvin time on the patient/control mixture in the early tests, is itself highly suggestive of either a heparin or heparin-like activity being present. Although it is possible that intravascular heparin may occur in certain clinical conditions in man, it is now being recognised increasingly that F.D.P. have anti-thrombic properties similar to heparin and can be partially or completely neutralised by protamine in vitro (Vreeken et al., 1966). Previous reports (Willoughby, 1963; Bloom and Campbell, 1965) of heparin-like anticoagulants in situations now known to be associated with D.I.C. would now seem more explainable on the presence of circulating F.D.P.

Cryofibrinogen is an intermediate product of fibrinogen-fibrin conversion. The presence of elevated levels of heparin precipitable fraction in the early stages of this haemorrhagic episode, is strong evidence for the presence of cryofibrinogen; and, if so, is pathognomonic of D.I.C. (Hardaway, 1966). Although there are many reports of cryofibrinogen occurring in animals with D.I.C., studies of its occurrence in man in similar situations are meagre. It can be seen that H.P.F. levels were markedly elevated above the normal values for the laboratory (44.5 ± 10.9 mgm./100 ml.) and remained so for up to 90 hours after delivery. However, without further study the results must be interpreted with caution and need not necessarily represent a response to D.I.C. For instance, the changes (if any) occurring of H.P.F. in patients with intra-uterine foetal death prior to delivery, and

following uterine evacuation operations, is not known and additional investigations are required.

In view of the marked activation of the fibrinolytic system as evidenced by the very low plasminogen levels and high F.D.P. levels it was notable that platelet counts were never greatly reduced. In seven patients with hypofibrinogenaemia secondary to interuterine death in whom there was severe bleeding (Pritchard and Ratnoff, 1955), the comment is made that marked thrombocytopenia did not occur, although in some patients, values were depressed below the lower limits of normal. There is little other reliable data in the literature and further studies of platelet levels would be of interest. Moderate thrombocytopenia is common in D.I.C. and its absence might indicate that the platelet levels were supranormal prior to the pathological process commencing. Platelet levels in the third trimester are, according to Ward and McArthur (1948) and Shaper et al. (1968), lowered, thus this former conclusion is less likely to be valid. The diagnosis of D.I.C., however, cannot be excluded by the mere absence of thrombocytopenia as demonstrated by Merskey et al (1967).

After therapy the decreased levels of clotting factors returned rapidly to normal but by 40-64 hours post delivery there was clear evidence of increased levels of these factors. The increases were not sustained and by 89 hours values were well within the normal ranges. Rachmilewitz et al. (1967) describe a patient who exhibited the defibrination syndrome followed by a hypercoagulable state and the development of multiple pulmonary emboli. Kropatkin and Izak (1968) report a similar case and although elevated levels of clotting factors are not always associated with thrombo embolic phenomena,

such incidents are more common when clotting factors are elevated than when they are in the normal range. It is also possible that the products of clotting released during the transformation of fibrinogen to fibrin stimulate not only an increase in fibrinogen but also of other clotting factors. Kropatkin and Izak (1968) infused thrombin into rabbits and were able to produce a hypercoagulable state in the post defibrination period and further experiments suggested that the effect could also be produced by the injection of the supernatant of clotted purified fibrinogen.

Hypofibrinogenaemia is by no means universal in patients with an intra-uterine death, and lowered levels of fibrinogen do not usually occur earlier than the fifth week after foetal death (Pritchard and Ratnoff, 1955). The mechanism by which the hypofibrinogenaemia arises is still not clear. Reid and Diamond (1953) and Schneider (1954) suggest that tissue thrombo-plastins from the degenerate foetus and placenta may enter the maternal circulation causing intravascular coagulation and consumption of fibrinogen. It is also possible that fibrinolytic activators from the macerated foetus pass into the maternal circulation and promote intravascular lysis of fibrin (Jürgens and Beller, 1959). However, Brakman and King (1965) made a detailed study of the fibrinolytic system in two patients but could not account for the decrease in fibrinogen by action of this system. Blood fibrinogen may also be precipitated progressively in the inter villous spaces of the placenta (Ashworth and Stouffer, 1956; Little and Phillips, 1962) thus reducing fibrinogen levels. It might be expected that if reduction of fibrinogen levels was dependent on the action of the fibrinolytic

system, levels of F.D.Ps. which reflect intravascular lytic activity, would be increased. In six patients studied in this laboratory with an average estimated time of intra-uterine death of five weeks, the F.D.P. levels were similar to those expected in the third trimester (Table 5:23). However, none of these patients were hypofibrinogenaemic. A prospective study of F.D.P. levels in a selected group of "high risk intra-uterine death" subjects would be of value. It might then be possible to assess the relevance of fibrinolytic activity to the decrease of fibrinogen levels.

In the present patient intra-uterine death had probably occurred almost ten weeks previously. It is thus possible that fibrinogen levels were low prior to operation and the surgical manipulation of the uterus had resulted in the release of tissue thromboplastins or lysins into the circulation with the resultant defibrination. Skjødtt (1967) was able to demonstrate, using a variant of the thromboplastin activation test as described by Astrup and Ollendorff (1961), that thromboplastic activity similar to the "serum thrombotic accelerator" of Wessler (1958), was present in the plasma of a patient who had sustained an intra-uterine death.

From Table 5:21 it can be observed that the heat precipitation method for assay of fibrinogen is not accurately reflecting the level of thrombin clottable fibrinogen. These results are similar to those obtained in the patient to be described next and provides further evidence for the unreliability of the assay method in certain clinical situations associated with high levels of F.D.P.

This patient was treated with a blood transfusion, a concentrate of fibrinogen and E.A.C.A. (Fig. 5:20). Fibrinogen and whole blood have

TABLE 5:23.

FIBRINOGEN AND F.D.P. VALUES IN SIX PATIENTS
WITH INTRA-UTERINE DEATH.

Subject	Fibrinogen mgm/100 ml.	F.D.P. μgm/ml.	Intra-Uterine Death (weeks)
1	200	14.0	6
2	354	13.6	7
3	228	5.9	5
4	285	10.0	3
5	190	14.0	4
6	400	14.0	4

been used frequently in this syndrome and the mortality is relatively low (McKay and Muller-Berghaus, 1967). Although E.A.C.A. was administered in this patient on the basis of greatly increased lytic activity demonstrable in the in vitro fibrinolytic tests, some authorities consider that the use of E.A.C.A. should be avoided if intravascular clotting is still proceeding. Spraitz et al. (1963) report one case in detail, treated with 6.0 gm. of fibrinogen and 4.0 gm. of E.A.C.A. The patient recovered but developed renal failure presumably secondary to non lysable fibrin thrombi in the kidney, and required dialysis on three occasions. In another patient with an intra-uterine death described by Rachmilewitz et al. (1967), multiple pulmonary emboli developed subsequent to E.A.C.A. therapy following an episode of D.I.C., although other factors were also operative in this patient. Pfeffer (1966), however, treated patients successfully with E.A.C.A. without untoward effects. Another approach to therapy is to administer heparin to decrease the rate of intravascular coagulation. Both Skjødtt (1967) and Lerner et al. (1967) report the successful use of heparin in patients with retention of a dead foetus and marked D.I.C. There was an increase of clotting factors, including fibrinogen, following heparin infusion and delivery was effected without the aid of either blood or fibrinogen. Phillips and Sciarra (1965) and Sherman and Middleton (1958) describe similar cases. In all these subjects, haemorrhage was not initially a presenting symptom although skin petechiae and ecchymosis were present. Lerner et al. (1967) state that if hypofibrinogenaemia and bleeding co-exist, heparin will not immediately reverse the D.I.C. and blood or fibrinogen should be used in the first instance. This situation was

applicable to the present patient.

In summary, this patient developed the typical features of disseminated intravascular coagulation following evacuation of the uterus for an intra-uterine death of 8-10 weeks. Treatment with whole blood, a concentrate of fibrinogen, and E.A.C.A. resulted in rapid complete recovery with an uneventful convalescence.

3. AMNIOTIC FLUID EMBOLISM.

Amniotic fluid embolism, a rare but serious complication of pregnancy, was first clinically described by Meyer (1926). Much more interest was taken in the disorder after the work of Steiner and Lusbaugh (1941), who demonstrated both experimentally and in pathological studies, the exact nature of the syndrome. Up until 1967, over 100 cases of this disorder had been reported in the English literature. (Anderson, 1967), and its incidence has been stated variably from 1:8,000 to 1:37,000 of live births (Steiner and Lusbaugh, 1941; Hemmings, 1947; Barno and Freeman, 1959). Although the incidence is thus relatively low, amniotic fluid embolism remains an important cause of maternal and foetal death. (Phillips, 1964; Russell and Jones, 1965). There are only seventeen cases of non-fatal presumed amniotic fluid embolism reported in the English literature, and amongst these patients some have been left with residual neurological defects.

The following case report is an account of a non-fatal but severe episode of disseminated intravascular coagulation occurring in mid-pregnancy and clinically presenting as an amniotic fluid embolism.

Case Report.

Mrs. E.B., a paragravida 4 + 1, aged 28, was seen at a gynaecological clinic with a request from her General Practitioner that termination of pregnancy should be considered. She was then four months pregnant, but had intermittently noticed slight vaginal bleeding with an associated brown discharge. There was also a history of some suprapubic pain but she had been well otherwise.

After admission to hospital subsequent investigations revealed that the vaginal discharge was minimal, pregnancy tests were positive, and the foetal heart could be heard. It was considered there were no grounds for termination of pregnancy but she remained in hospital for observation.

Progress over the following week was satisfactory; the pain settled and the vaginal discharge was much less. Foetal movements were felt. There were no other clinical symptoms and she was apyrexia.

Suddenly at 11.0 p.m., while sitting in bed knitting, the patient collapsed. She was seen by the staff almost immediately and found to be unconscious and breathing stertorously. There was cyanosis and the blood pressure was unrecordable. Further examination revealed a tachycardia with a gallop rhythm and multiple extra systoles. Numerous scattered rhonchi were heard over both lung fields and the jugular venous pressure was raised. Oxygen administration was commenced and the blood pressure rose to ¹⁰⁰/40 and the patient recovered consciousness. She complained of severe lower abdominal pain and on examination the uterus was hard and tender. Slight fresh vaginal bleeding was now noticed. There was no

evidence of calf tenderness or pain.

An electrocardiograph confirmed the presence of a tachycardia with multiple extra systoles and evidence of right heart strain. A portable chest X-ray showed patchy changes in both lung fields and particularly in the right mid zone, with some dilatation of the right side of the heart, the appearance being consistent with multiple pulmonary emboli. A sample of venous blood obtained was incoagulable, and further coagulation investigations revealed a complete afibrinogenaemia with evidence of increased fibrinolysis. Evidence of a slight metabolic acidosis was present with a $p\text{ CO}_2$ of 12 mEq/L.

The foetal heart could not now be heard and as it was considered that abortion might occur, preliminary correction of the coagulation defect was advised. Vaginal bleeding was still only minimal, but some bruising had occurred at venepuncture sites. The patient's general condition was otherwise improving.

Two-and-a-half hours after the initial episode an infusion of 2.5 gm. of E.A.C.A. was commenced followed by 4.0 gm. of Cohn Fraction I (60% clottable protein). Diamorphine was also administered for abdominal pain. Bleeding was still minimal. A similar E.A.C.A./fibrinogen infusion was repeated over the next two hours and the fibrinogen level seven hours post collapse rose to 102 mgm./100 ml. Her general condition continued to improve with an increase of both blood pressure and pulse rate. A further 1 gm. of E.A.C.A. and 4.0 gm. of Cohn Fraction I was continued by intravenous infusion.

By ten hours post collapse her condition was good. The slight

vaginal bleeding had ceased and the abdominal pain had disappeared. Full coagulation studies, summarised in Table 5:24 demonstrate that as well as an afibrinogenaemia there had been a decrease of platelet levels, coagulation Factors V (proaccelerin) and VIII (antihaemophilic factor), and a marked increase in the level of fibrin/fibrinogen degradation products. A diagnosis of disseminated intravascular coagulation, presumably secondary to an amniotic fluid embolus, was made. Subsequent coagulation tests after therapy demonstrated improvements in all parameters measured. Urine had been passed and it was noticed that it was heavily laden with albumin and was discoloured red. There was no sign of labour commencing and on vaginal examination the cervical os was not dilated. Small doses of E.A.C.A. and fibrinogen were continued for another five hours and therapy was then discontinued.

It was decided that the uterus should be emptied without delay, but that an oxytocin drip would be inadvisable. An abdominal hysterotomy, under cover of fibrinogen, was therefore performed 82 hours after the initial acute collapse.

At operation the uterus was of size appropriate to that of the calculated gestation period and was of normal appearance. On opening the uterus no liquor was present and the dead foetus was removed. The placenta was firmly adherent to the uterine wall and no significant retroplacental clot was present. There was no difficulty with haemostasis and immediate post operative progress was satisfactory.

Two days after the operation she complained of right side lower chest pain, made worse on inspiration. Chest X-ray was clear and E.C.G.

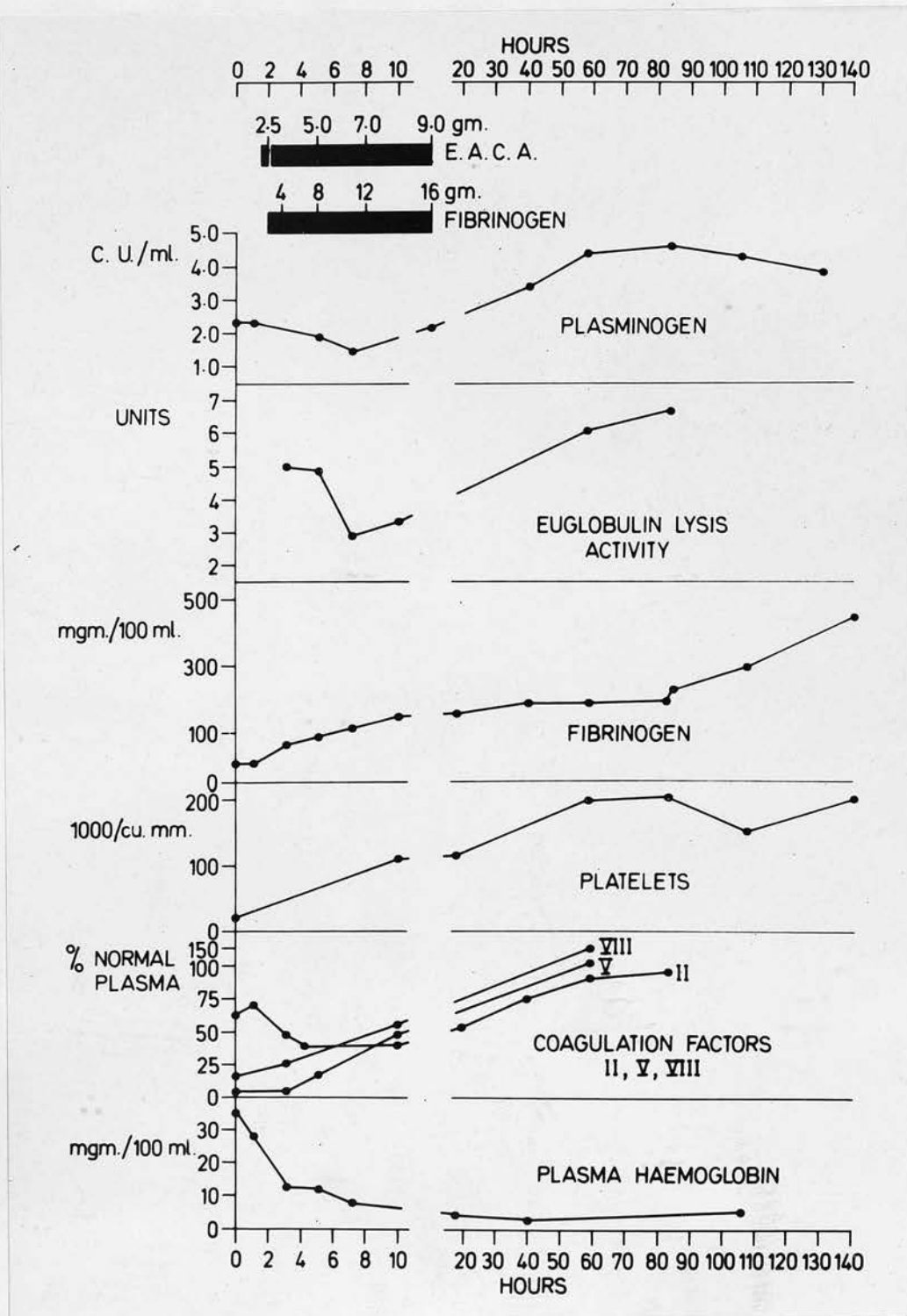


Fig. 5:22. Results of laboratory investigations on Mrs. E.B.
(amniotic fluid embolus)

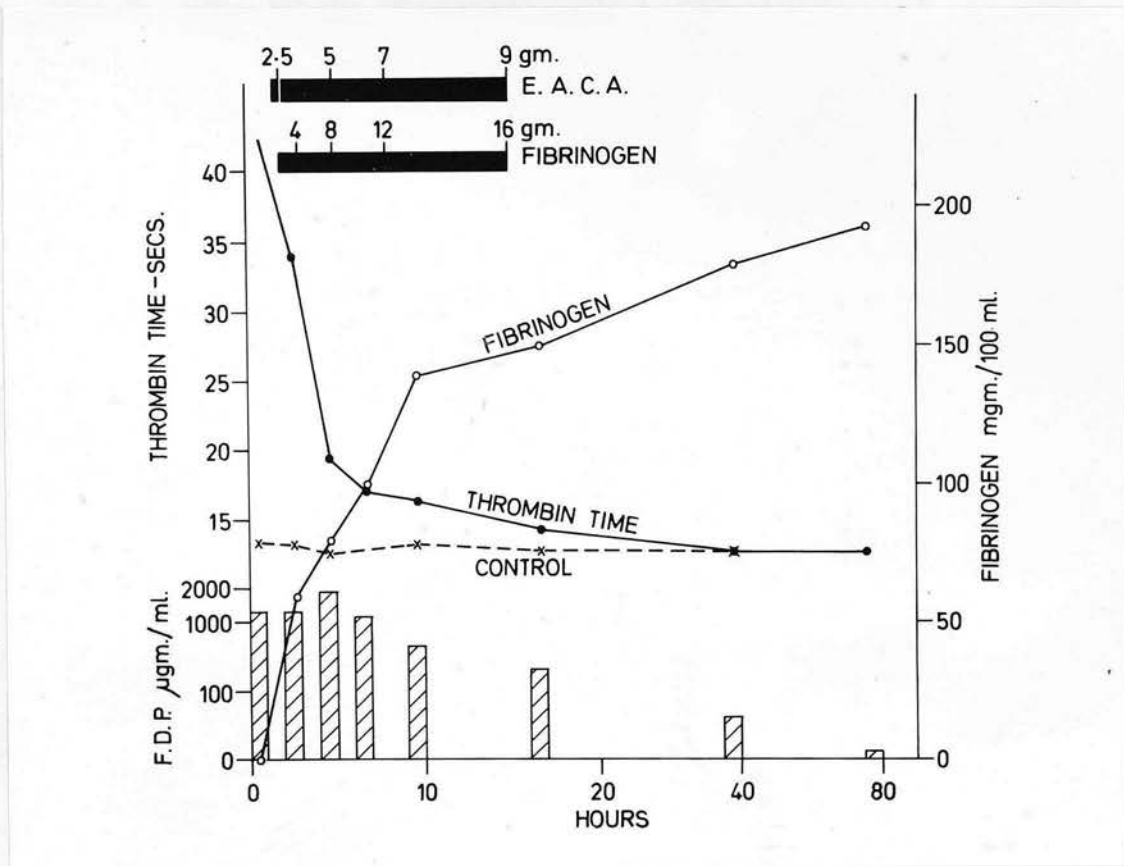


Fig. 5:23. Further results of investigations on Mrs. E.B.
(amniotic fluid embolus)

TABLE 5:24.

RESULTS OF COAGULATION AND FIBRINOLYTIC STUDIES ON MRS. E.B. (AMNIOTIC FLUID EMBOLUS)

Hours	Fibrinogen (mgm/100 ml)	F.D.P. (μgm/ml)	E.L.T. (units)	Fibrin Plates (mm ²)		Plasminogen c.u./ml.	Platelets 103/cmm.	Factor II %	Factor V %	Factor VIII %	Factor IX %	Factor X %	K.C.C.T. (secs.)		Prothrombin Time (secs.)	
				Euglobulin	Plasma Assays								Test	Control	Test	Control
0	0	1572	No clot	-	425	2.25	35.0	60	5	15	100	85	-	41	-	14
1	0	1311	-	-	-	2.25	-	67	-	-	-	-	-	-	-	-
3	61	1290	5.00	100	72	-	-	48	5	24	103	92	74	40	27	14
5	80	1894	4.83	483	105	1.99	-	38	15	-	-	-	54	40	21	14
7	102	1125	2.77	-	-	1.50	-	-	-	-	-	-	-	-	-	-
10	140	643	3.19	279	45	-	105.0	41	49	50	-	93	38	45	17	13
17	147	308	-	-	-	2.06	110.0	51	-	-	-	96	-	-	15	14
40	179	60	-	-	-	3.16	-	75	-	-	-	-	38	35	-	-
58	188	30	5.99	420	-	4.34	200.0	81	102	147	-	123	-	-	-	-
82	194	15	-	-	-	-	200.0	95	-	-	-	-	33	40	-	-
84	225	-	6.57	506	223	4.51	150.0	-	-	-	-	-	-	-	-	-
106	296	10	-	-	-	4.15	150.0	-	-	-	-	-	29	33	15	13
130	436	-	-	-	-	3.75	200.0	-	-	-	-	-	29	40	-	-

TABLE 5:25.

CALCIUM-THROMBIN AND CALCIUM-ARVIN TIMES ON MRS. E.B. (AMNIOTIC FLUID EMBOLUS)

Hours	Calcium-Thrombin Time (secs)			Calcium-Arvin Time (secs)			Calcium-Thrombin Time and Protamine		
	Test	Control	Mixture	Test	Control	Mixture	Test	Control	Mixture
1		13.2	17.2		15.1	14.3	120+	9.2	9.6
3	34.0	12.7	17.6	23.8	15.5	15.8	13.4	8.9	10.1
5	19.4	12.5	16.2	17.2	15.6	16.4	9.9	8.8	-
7	17.0	12.6	14.0	17.4	16.8	16.6	9.8	9.2	8.3
10	16.6	13.2	14.9	16.1	16.7	15.5	9.4	10.6	11.1
17	14.3	12.7	12.5	14.5	16.9	15.7	10.7	10.1	10.0
40	12.4	12.3	12.4	-	-	-	-	-	-
82	12.3	12.3	-	16.5	16.8	-	-	-	-
C L O T T I N G M I X T U R E S									
	0.1 ml. Tris buffer	0.1 ml. Tris buffer	0.1 ml. Tris buffer	0.1 ml. Tris buffer	0.1 ml. Tris buffer	0.1 ml. Tris buffer	0.1 ml. Protamine 10 mgm/ml.		
	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma	0.1 ml. Test or Control plasma		
	0.1 ml. Ca/Thrombin	0.1 ml. Ca/Thrombin	0.1 ml. Ca/Thrombin	0.1 ml. Ca/Arvin	0.1 ml. Ca/Arvin	0.1 ml. Ca/Arvin	0.1 ml. Ca/Thrombin		

showed no specific changes. The symptoms settled spontaneously and the remainder of her convalescence was uneventful. She was discharged two weeks later in good health.

Laboratory results are tabulated in Tables 5:24-27 and Figs. 5:22-25.

Comments.

To establish the definitive diagnosis of an amniotic fluid embolus access to autopsy material is required to demonstrate the presence of squames from amniotic fluid or meconium mucus in the lungs or in the blood from the right side of the heart. However, the syndrome should be suspected clinically when sudden collapse and shock occur before, during and after labour, especially if accompanied by pulmonary and haemorrhagic manifestations (Scott, 1963). In this patient, the diagnosis could not fortunately be established with certainty and although the clinical picture was consistent with the diagnosis of an amniotic fluid embolus several unusual features were present.

In a survey of 32 cases of proved amniotic fluid embolism (Anderson, 1967) the duration of gestation varied from 38 to 44 weeks. Despite a careful search of the literature, cases of amniotic fluid embolism occurring in mid-pregnancy have not been found. The present patient was between 16 and 18 weeks pregnant and labour had not commenced. Although this might be indicative of an alternative diagnosis other clinical conditions producing such alarming clinical and haematological findings are even less likely. Multiple pulmonary emboli from thrombi in the legs or pelvis might explain the pulmonary findings but are unlikely to produce such a

severe complete defibrination. There was also no clinical history or physical evidence of deep vein thrombi. In view of the slight vaginal discharge prior to the episode, infection may have been a factor. However, the history is quite unlike that usually seen in septic abortions or chorioamnionitis. In this patient the foetus was alive, there was no pyrexia, and the patient was quite well. Culture of the vaginal discharge was, however, not performed. It is also unlikely, as the patient had been in hospital under observation for a week prior to this acute episode, that there had been any physical interference with the pregnancy. The symptoms could also not be explained on heart disease as no abnormalities were detected when the patient was subsequently examined by a cardiologist. There was no history of Mendelson's syndrome due to inhaled vomit. It was therefore still clinically possible that an amniotic fluid embolus had occurred, even at the relatively early stage of pregnancy.

Amniotic fluid emboli do not usually occur without some evidence of labour. Anderson (1967) notes four cases without labour occurring late in pregnancy but the remaining 28 subjects described were all in labour. The typical case history described in the literature is that of an elderly woman of high parity, late in gestation, who during labour develops the signs and symptoms of sudden embolism. The use of oxytocic stimulation of the uterus has also been implicated in the pathogenesis of the disorder (Anderson, 1967) although the statistical evidence to support this supposition is lacking. Neither labour nor the use of oxytocins were relevant features in this patient.

The route by which amniotic fluid enters the maternal circulation

has not clearly been defined. Leary and Hertig (1950) found foetal squames in abnormal locations within the normal placenta and membranes after uncomplicated deliveries. They deduced that transient tears in the chorionmniotic membrane permitted fluid to enter the maternal circulation through subplacental sinusoids. Josey (1961, 1966) suggested that amniotic fluid enters the circulation by way of lacerated venous sinuses in the lower segment of the uterus. He describes several patients with hypofibrinogenaemia in whom there was detected significant rupture of the lower segment of the uterus. Further evidence comes from Landing (1950) who demonstrated that amniotic fluid could be introduced into the circulation by way of abnormally opened myometrial or placental vessels in such conditions as ruptured uterus or placenta accreta. Schneider and Moya (1961) indicated that it was probable that the increased intra-uterine pressure during labour would tend to drive amniotic fluid towards the lower segment and cervix, thus perhaps facilitating infusion of fluid into the maternal circulation. Such an explanation is unlikely in this patient as labour was not in progress.

The syndrome of amniotic fluid embolism is generally believed to be due to the embolization of amniotic fluid particulate material (Steiner and Lusbaugh, 1941; Cron et al., 1952) or maternal fibrin which has resulted from the thromboplastic activity of the infused amniotic fluid (Weiner, 1950) or possibly platelet thrombi (Brozman, 1961). Although Thompson et al. (1966) found that two separated infusions of filtered amniotic fluid produced an augmented decrease of fibrinogen in dogs, most authorities now accept that an allergic or anaphylactic mechanism probably does not play a

significant part in the pathogenesis of the syndrome. Tio (1956) infused clarified amniotic fluid in volumes of 5-500 ml. into 73 human subjects with no untoward results, and similar findings in animals have been reported by MacMillan (1968) and Stolte et al. (1967).

This concept of amniotic squames and meconium being responsible for the clinical manifestations of amniotic fluid embolisation would be at variance to the picture seen in Mrs. E.B. There is a paucity of squames in amniotic fluid prior to the 32nd week of pregnancy (Josey, 1966) so it is less likely that the pulmonary symptoms were due to embolisation of particular material. MacMillan (1968) also noted that the severity of embolic effects appeared to be related to the number of cells infused (in rabbits) but did not regard the embolic effects of amniotic fluid sufficient alone to account for sudden death during labour. The volume of amniotic fluid between 16 and 20 weeks ranges between 250-290 ml. (Hyttén and Leitch, 1964) or even slightly lower (Rhodes, 1966) and this volume of fluid, even if infused rapidly would appear unlikely to produce severe respiratory embarrassment and collapse. The diagnosis of amniotic fluid embolus must therefore be a tentative one held in the absence of an adequate alternative explanation of the dramatic findings.

The clinical onset of an amniotic fluid embolism is characterised by the sudden development of acute shock accompanied by severe dyspnoea, cyanosis and often unconsciousness. Cron et al. (1952) reported that when liquor, rich in squames was injected into rabbit ear veins, pronounced dyspnoea and sudden death occurred. When the same liquor was injected into the femoral artery shock was not produced and the animals survived.

They therefore deduced that it was the particulate matter of amniotic fluid that caused the pulmonary clinical symptoms and radiological changes. These views have been supported by most other workers and it is thought that the mechanical obstruction in the distal pulmonary tree results in vagus mediated vaso constriction of both pulmonary and coronary arteries, with a consequent fall in blood flow, and cardiac output resulting in hypotension. There is also often enlargement of the right side of the heart, due to pulmonary hypertension and radiological opacities in the lung field are common (Scott, 1963). Electrocardiographic changes also occur (Arnold et al., 1961) and usually non specific changes compatible with right heart strain are present. The present patient demonstrated most of these clinical, radiological and electrocardiographic changes, and the findings were quite consistent with a diagnosis of amniotic fluid lung emboli. The slight metabolic acidosis would also be in keeping with this diagnosis.

Although the aetiology of the initial collapse was debatable there could be no doubt that in this patient a massive episode of disseminated intravascular coagulation had occurred. There was afibrinogenaemia, a marked decrease in the assayed level of several clotting factors, prolongation of the clotting times in several clotting tests, thrombocytopenia, increased levels of F.D.P. and decreased plasminogen levels (Table 5:24). Despite these marked changes haemorrhage was minimal. Improvements in most of these parameters occurred after therapy was commenced.

Blood incoagulability occurs in as many as 40% of patients who survive the first hour after the initial episode (Aguillon et al., 1962). It is thought to be due to the thromboplastic activity of cellular components

from the amniotic fluid resulting in the formation of fibrin and consumption of fibrinogen. This process is enhanced by the activation of the fibrinolytic system either by a direct effect of tissue activator released from the amniotic cells or by a secondary compensatory reaction as a result of the excess fibrin formation. Plasminogen, converted to plasmin, acts proteolytically on fibrin and fibrinogen as well as on several clotting factors, thus depleting these haemostatic factors even further. It is also possible that platelet aggregation around particulate material in the pulmonary arterioles and capillaries with consequent release of platelet-factor-3 may enhance the activity of the coagulation system. However, despite marked alterations in haemostatic mechanism parameters, bleeding externally or into the skin was not present in this patient and our knowledge of the factors maintaining vascular integrity is thus still very probably far from complete.

The value of the assay of F.D.P. can clearly be seen. Concentrations of degradation products reached high levels and then decreased subsequent to therapy. The recent introduction of the rapid assay of F.D.P. as described earlier in this thesis will undoubtedly facilitate the identification of lytic states, and thus provide more adequate knowledge on which to base therapy. In conjunction with the F.D.P. assay, immunoelectrophoretic investigations of the series of pathological plasmas and sera from Mrs. E.B. was performed (Figs. 5:24 and 25). The initial immunoelectrophoretic patterns closely resemble that produced by the in vitro digestion of fibrinogen by either plasmin (Fig. 5:24) or urokinase (Fig. 5:24). In the following slides from Mrs. E.B. the disappearance of

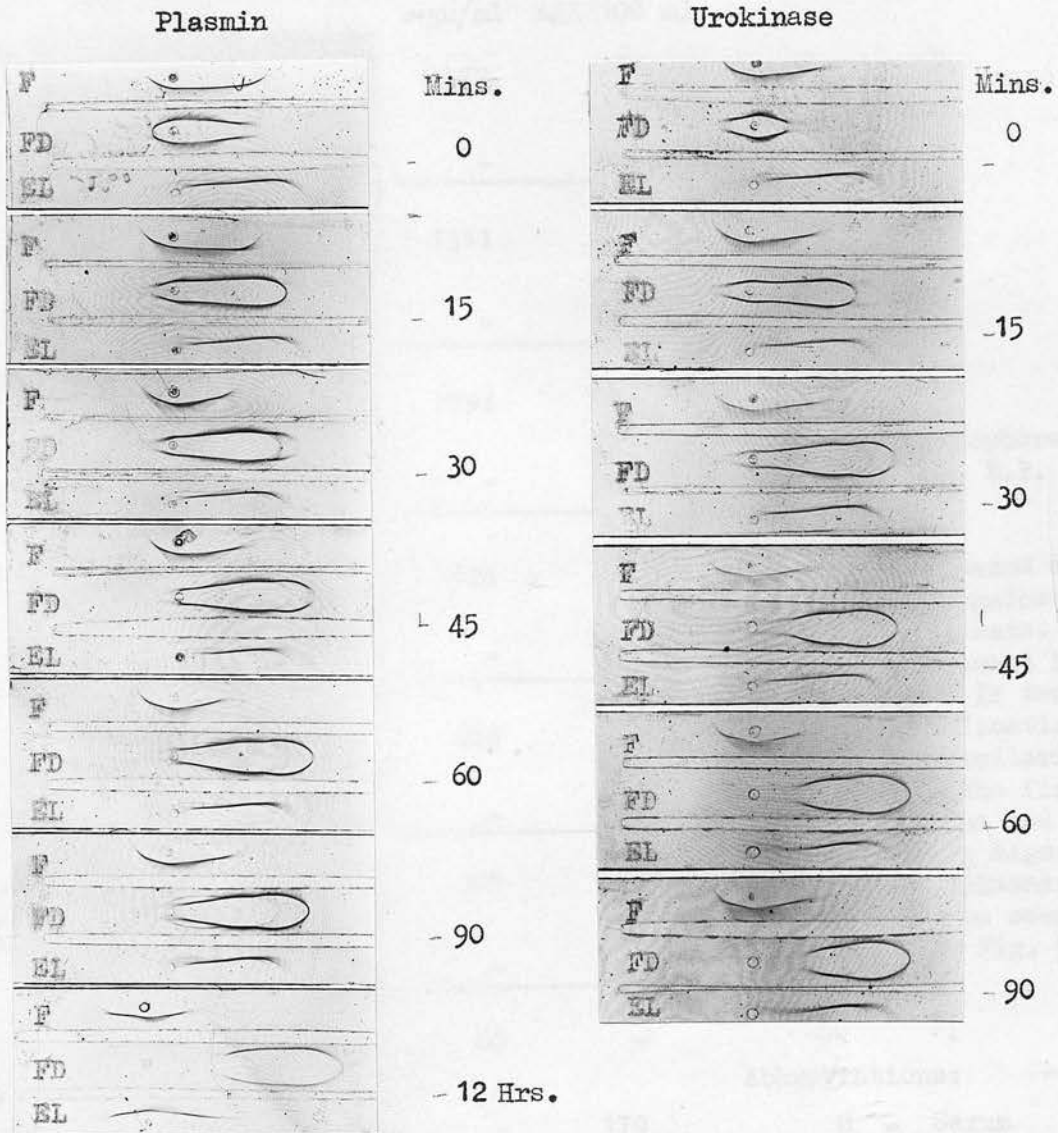


Fig. 5:24. The in vitro digestion of fibrinogen by plasmin or urokinase (courtesy of Dr. P.C. Das and Dr. I.M. Riding). The lytic products of fibrinogen can be identified in the centre block of each immunoelectrophoretic slide.

Abbreviations:

F = Normal Fibrinogen
 FD = Fibrinogen Digest
 EL = Euglobulin Lysate

Hrs.		F.D.P. TRCHII μgm/ml	Fibrinogen mgm/100 ml
0	S	1572	-
	P	-	0
1	S	1311	-
	P	-	0
5	S	1894	-
	P	-	80
7	S	1125	-
	P	-	102
10	S	643	-
	P	-	140
17	S	308	-
	P	-	147
40	S	60	-
	P	-	179
58	S	30	-
	P	-	188
82	S	15	-
	P	-	194
106	S	10	-
	P	-	296

Fig. 5:25.

Immunoelectrophoretic (IE) studies on Mrs. E.B. (amniotic fluid embolus).

Plasma and serum samples were each run against a fibrinogen antisera. There is a good agreement between the TRCHII and IE techniques for the identification of F.D.P. The similarity of the F.D.P. in the first four IE slides to the end products of the in vitro digestion of fibrinogen by plasmin or urokinase can be seen (as illustrated in Fig. 5:24).

Abbreviations:

S = Serum
P = Plasma

F.D.P. from both plasma and sera can be seen clearly, and can be correlated with both the level of fibrinogen and F.D.P. (as assayed by the T.R.C.H.I.I.)

Fibrinogen assay results, as determined by three separate methods, are tabulated in Table 5:26. It is at once apparent that the heat precipitation method (Thorpe Nephelometer) is an inaccurate technique in the presence of high levels of F.D.P. There is, however, a good correlation between the other two methods of fibrinogen assay. Fibrinogen assays that rely on the physico-chemical properties of fibrinogen may yield misleading results in patients with the defibrination syndrome as evidenced by the work of Sharp et al. (1958) using an ammonium sulphate turbidity measurement. It is also known that methods of fibrinogen determination that rely on heat precipitation can be unreliable in the presence of fibrinogen derivatives as these share the property of precipitation with fibrinogen (Fletcher et al., 1966). The present results would be in accord with these findings but the good correlation of fibrinogen values between the other two methods is in agreement with the studies of Merskey et al. (1967).

Serial studies clearly demonstrate the marked improvement in the laboratory tests following therapy. Fibrinogen concentrate and E.A.C.A. in small doses was administered as a precaution against premature labour commencing. In the initial blood specimen there was evidence of increased lytic activity on the fibrin plate assay system, indicative of either residual plasmin or plasminogen activator, and it was thought that this activity should be reduced before fibrinogen was infused. Plasminogen levels did not immediately increase after therapy and prothrombin levels apparently fell. This might indicate that intravascular coagulation was

TABLE 5:26

RESULTS OF FIBRINOGEN ASSAYS BY THREE DIFFERENT METHODS

ON MRS. E.B. (AMNIOTIC FLUID EMBOLUS)

Hours	Ellis & Stransky (mgm./100 ml.)	Ratnoff & Menzies (mgm./100 ml.)	Thorpe Nephelometer (mgm/100 ml.)	F.D.P. (gm/ml.)
0	0	10.0	180	1,570
1	0	7.5	173	1,311
3	61	70.0	194	1,290
5	61	100.0	215	1,894
7	102	110.0	205	1,125
10	140	128.0	179	643
17	147	146.0	144	308
40	179	184.0	100	60
58	188	192.0	134	30
82	194	-	-	15
84	225	-	-	-
106	296	-	-	10
130	436	-	-	-

continuing and this conjecture might be supported by the even further increased level of F.D.P. after fibrinogen and E.A.C.A. therapy. Although it was not considered the most reasonable therapy at the time, it would have been of considerable theoretical interest to see the effect of E.A.C.A. and heparin alone in this type of obstetrical situation.

The thrombin times are of some interest. From Table 5:25 it can be seen that the low levels of fibrinogen are prolonging both the Ca-thrombin and Ca-Arvin times up to Hour five. However, the 1:1 mixture of test plasma and control plasmas show that although the Ca-thrombin time was still prolonged even when fibrinogen levels are normal, the Ca-Arvin time on the mixture was normal. This is strong evidence for heparin-like activity being present. In this situation F.D.P. was probably exerting antithrombin effect, as Arvin is unaffected by the presence of F.D.P. or heparin. It can also be seen that the addition of protamine (10 mgm./ml.) is correcting the thrombin time at the seven hour mark. Before this stage only a small effect is present. Protamine has been shown to correct long thrombin times associated with the presence of F.D.P., but much larger amounts are required to neutralise heparin (Vreeken et al., 1966). Willoughby (1963) used protamine in vivo in a haemorrhagic state in the puerperium associated with defibrination with temporary cessation of bleeding. It is possible that varying molecular weight sizes of F.D.P. may act in different manners with protamine and further studies would be of great interest.

One of the most interesting facets of this case study was the appearance of haemolysis in specimens removed before fibrinogen or E.A.C.A. was administered. Plasma haemoglobins and haptoglobin levels are tabulated

TABLE 5:27

RESULTS OF PLASMA HAEMOGLOBINS AND HAPTOGLOBINS

ON MRS. E.B. (AMNIOTIC FLUID EMBOLUS)

Hour	Plasma Haemoglobin mgm. %	Haptoglobins mgm. %	Haemoglobin %
0	35	0	82
1	28	0	-
3	12	-	-
5	12	-	-
7	8	-	69
17	4	-	68
40	3	-	78
82	-	30	-
106	6	50	76
Normal Range	0 - 4	30 - 200	100

in Table 5:27 and Fig. 5:23. Loeliger (1966) mentions severe acute haemolytic anaemia occurring in conjunction with amniotic fluid embolism and Hart (1966) comments on the appearance of intravascular haemolysis in such varied clinical conditions as gastric carcinoma, haemolytic-uraemic syndrome and eclampsia, all of which were associated with D.I.C. Studies by Brain and Beck (1965) suggest that the haemolysis occurring in the syndrome of disseminated intravascular coagulation may be due to trapping of red cells in microthrombi which accumulate temporarily in small blood vessels. Using an elegant cine microphotography technique these workers have been able to demonstrate that in in vitro models, fibrin networks, in a moving stream of blood can distort and damage red cells with the consequent release of haemoglobin (Rubenberg et al., 1967; Bull et al., 1968). This attractive theory is supported by sporadic case reports in which haemolysis and intravascular coagulation have been associated (Hart, 1966; Loeliger, 1966; Baker and Brain, 1967; Schneider, 1968). The present patient exhibited similar features with macroscopic haemolysis being visible in the specimens taken immediately after the initial collapse. The changes in plasma haemoglobin were fortunately not complicated by the infusion of blood.

It can also be seen from Table 5:27 that red cell haemoglobin levels fell appreciably during the acute episode, despite no marked vaginal haemorrhage. Renal failure did not develop in this patient but it was observed that the urine on the first occasion after the embolus was red in colour and contained considerable protein. This may have represented renal excretion of haemoglobin but unfortunately the specimen was not

specifically investigated. Disturbances of the renal function are not uncommonly associated with amniotic fluid emboli (Scott, 1963) but no reports of renal cortical necrosis appear in the literature, this perhaps being a reflection of the high immediate mortality of the disorder. Fibrin thrombi in the kidneys have, however, been reported in the kidneys at autopsy (Tuller, 1957).

This case history thus demonstrates the main changes occurring in the haemostatic mechanism in a situation associated with severe disseminated intravascular coagulation. Successful therapy of such an emergency depended on the prompt recognition and subsequent therapy of the underlying condition, coupled with adequate coagulation and fibrinolytic investigations as a guide to both diagnosis and subsequent progress.

4. TERMINATION OF PREGNANCY.

Therapeutic abortion using intra-amniotic hypertonic solutions has become a commonly accepted procedure in many hospitals and the history of the method has been reviewed by Kerr et al. (1966). The simplicity of the technique coupled with the low incidence of complications and high success rate has further encouraged its use (Menzies and Hawkins, 1968). However, termination of pregnancy is still not without risk. In series studied in Denmark and Sweden, the incidence of maternal death is 2:1,000 (Oram, 1952), and there is a morbidity rate of 5 - 15% (Berthelsen and Ostergaard, 1959).

Complications of this relatively new method of therapeutic abortion are now becoming available. Wagatsuma (1965) reported 25 deaths

after intra-amniotic injection and two deaths have been reported in Britain (Cameron and Dayan, 1966). Goldstein (1968) recently reported the occurrence of a probable amniotic fluid embolus after an injection of intra-amniotic saline.

The following case report outlines further similar complications of this method of inducing abortion.

Case Report.

Mrs. R.S., aged 31, was admitted to a peripheral hospital for termination of pregnancy on the grounds of severe endogenous depression. She had had two previous uncomplicated pregnancies and there was no significant medical history of note. The pregnancy had advanced to the 21 week gestation stage and she had been quite well. Soon after admission 200 ml. of 20% hypertonic saline was injected into the amniotic sac, after prior removal of a similar volume of amniotic fluid. Labour did not commence immediately and 18 hours later she was noted to be pyrexia (102°F) and was started on Crystamycin. Uterine contractions became evident.

During the labour the patient became unduly distressed and brisk haemorrhage commenced. Hypotension was marked and the initial blood loss was estimated at 500 ml. Delivery was effected and it was noted that blood taken for matching did not clot.

With supportive measures there was some improvement in blood pressure but bleeding continued and a tachycardia (120/min.) remained. In view of the blood loss three bottles of blood was infused as well as 4 gm. of fibrinogen. Blood pressure improved rapidly on this regime with cessation of haemorrhage. She subsequently made an uneventful recovery. On discharge she was in good

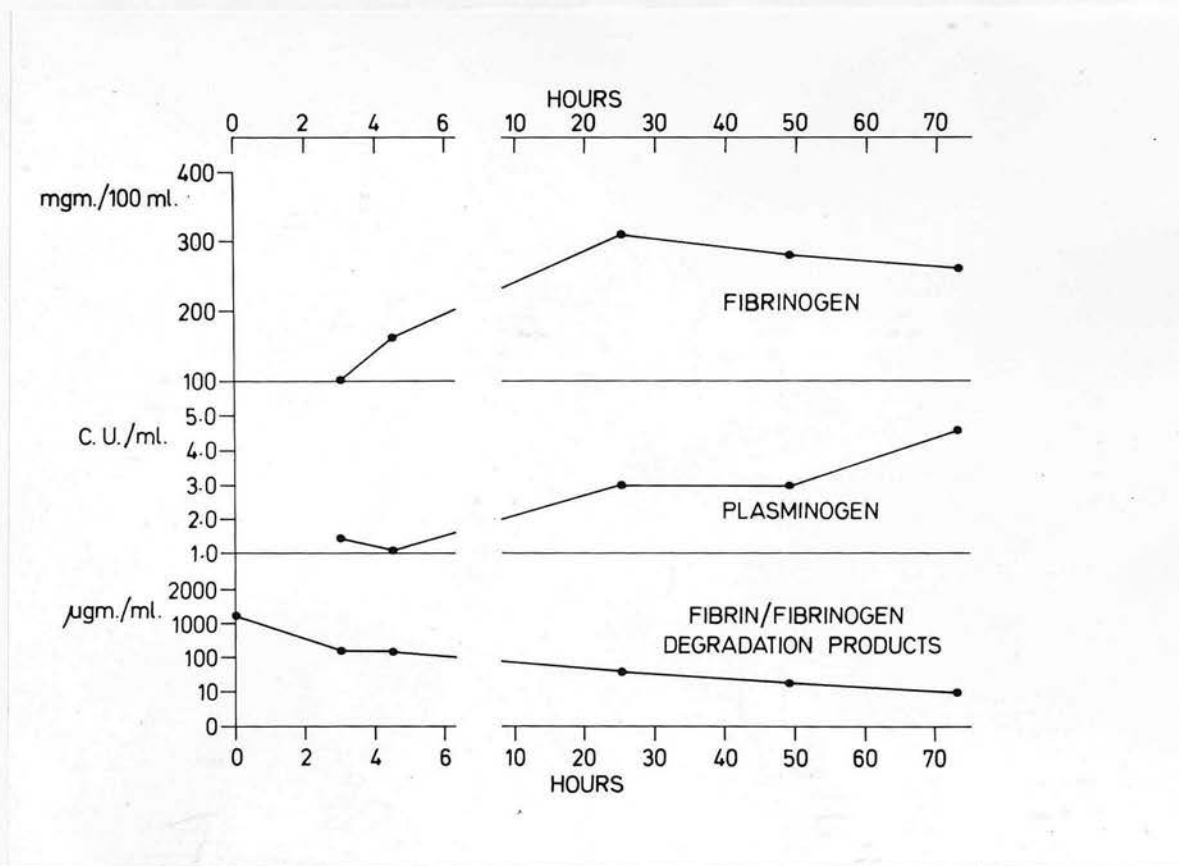


Fig. 5:26. Results of investigations on Mrs. R.S. (termination of pregnancy)

TABLE 5:28¹RESULTS OF COAGULATION AND FIBRINOLYTIC INVESTIGATIONS
ON MRS. R.S. (TERMINATION OF PREGNANCY)

Time Hours	Fibrinogen mgm/100 ml	Plasminogen C.U./ml	F.D.P. gm/ml.	Platelets per cmm.
4	-	-	1167.0	95,000
7	101	1.51	292.0	-
9	165	1.14	292.0	-
25	310	3.05	60.8	-
49	280	3.02	36.5	-
73	261	4.61	9.8	170,000

health, apart from a haemoglobin of 68%

The laboratory findings are summarised in Table 5:28 and Fig. 5:26

Comment.

The laboratory findings in this patient are consistent with an episode of disseminated intravascular clotting. There was a lowered level of platelets, a hypofibrinogenaemia, very low plasminogen levels, and a marked elevation of F.D.P. Specific coagulation assays were not performed although whole blood clotting times done at the bedside were within normal limits.

The aetiology of the event is less clear. In view of the pyrexia it is possible that sepsis associated with the retained products of conception was a factor, although premature separation of the membranes or a small amniotic fluid embolus cannot be excluded. It also cannot be proved that the method of induction of abortion was related to the pathological findings. The association is, however, suggestive and again points clearly to the potential risk in such procedures. This patient may not have survived in situations where blood transfusion facilities were not available quickly.

It can be seen that plasminogen levels did not increase rapidly after the episode and by nine hours the level was still greatly decreased, despite the infusion of plasminogen contaminated fibrinogen. This phenomena was also noticed in other of the subjects (2 and 3) studied and might suggest that either the production of plasminogen is delayed or utilization for lytic activity is still proceeding. In situations where intravascular coagulation is still continuing, the administration of fibrinogen may further promote the continuation of the disorder. Skøjdt (1967) advises the use

of fibrinogen infusions only after delivery when the risk of the passage of tissue thromboplastin to the maternal circulation is eliminated. McKay and Müller-Berghaus (1967) also warn of the theoretical risk of fibrinogen therapy when procoagulant agents are still acting in vivo as there is an increased risk of further fibrin deposition in susceptible organs. However, it is difficult to identify by presently available laboratory techniques, procoagulant substances in plasma and clinical judgement is required in the use of fibrinogen on the basis of a general knowledge of the disease process. One possible compromise method of therapy would be to administer fibrinogen and heparin together; one to correct any deficiency and the other to decrease the rate of activity of remaining residual thromboplastic substances.

This patient did not require the administration of E.A.C.A. as an adequate response was obtained by the use of whole blood and fibrinogen. Although E.A.C.A. is a useful antifibrinolytic drug its use in situations associated with continuing intravascular clotting can be hazardous. After E.A.C.A. treatment fibrin clots are preserved beyond the time they would be lysed spontaneously and the protective effect of the fibrinolytic system in clearing the vascular bed is thus greatly decreased. Several reports of bilateral renal cortical necrosis and thrombi in other organs have appeared following its use (Naeye, 1962; Andersson, 1963; Stamm et al., 1963; Nilsson et al., 1966).

In the acute phase of this illness, three bottles of blood and 4 gm. of fibrinogen were required for clinical resuscitation. It is possible that the blood loss which occurred before treatment was commenced was underestimated as the haemoglobin level on discharge was only 68%. This perhaps

underlines the severity of the defibrination process as the haemoglobin before delivery was known to be 96%.

This case study underlies the protean nature of the syndrome of disseminated intravascular coagulation. It can occur in a variety of obstetrical situations and unless the clinician is alert to its detection unnecessary mortality and morbidity may occur. Once evidence of its presence is detected therapy must be rapid and judged carefully, and the value of adequate coagulation and fibrinolytic investigations are self evident.

5. SECONDARY POST-PARTUM HAEMORRHAGE.

This case report is presented as an unusual example of post-partum haemorrhage in which a fibrinolytic process was apparently maintained for a prolonged period without recognition.

Case Report.

Mrs. M.S., a 29 year old para-gravida I was admitted to the Simpson Memorial Maternity Pavilion with a diagnosis of a secondary post-partum haemorrhage. Ten days prior to admission she had delivered at home a full term normal male babe. Blood loss was said to be marked at this delivery. Lactation had been suppressed with Stilboestrol but on the fourth day of the puerperium she was started on phenylbutazone, 100 mgm. t.d.s. for a superficial thrombophlebitis behind the right knee. This treatment had been continued up to the day of admission, as had been the Stilboestrol. Prior to admission she had started passing blood clots and continued to bleed in spite of the administration of ergometrine by her General Practitioner. The

uterus was also noted to be enlarged up to the umbilicus.

On initial examination her blood pressure was $100/60$; pulse 90; and she was apyrexia. Blood was matched and she was started on a syntocinon drip. The uterus was evacuated under general anaesthesia but no definite placental tissue was found. She continued to bleed briskly. Blood pressure fell and pulse rate increased despite additional ergometrine, antibiotics and transfusion of four bottles of blood. Clotting investigations demonstrated increased fibrinolytic activity associated with hypofibrinogen-aemia and 4 gm. of Cohn Fraction I was therefore administered as well as 2.5 gm. of E.A.C.A. She improved slowly on this therapy and in all was given 8 gm. of Cohn Fraction I and 5.0 gm. of E.A.C.A.

She remained well, if rather apprehensive, for the next few days but on the sixth day post operation she had another small vaginal bleed which was not of sufficient note to warrant therapy. She was finally discharged ten days post operatively, with a haemoglobin of 86% and apparently well.

On a subsequent visit six weeks later she had remained in good health with no recurrence of the bleeding.

The coagulation and fibrinolytic results are recorded in Table 5:29 and Fig. 5:27.

Comments.

This is an unusual case history and the aetiology of the initiating disorder can only be conjectured. The puerperium was complicated by both a primary post-partum haemorrhage and the development of a thrombophlebitis,

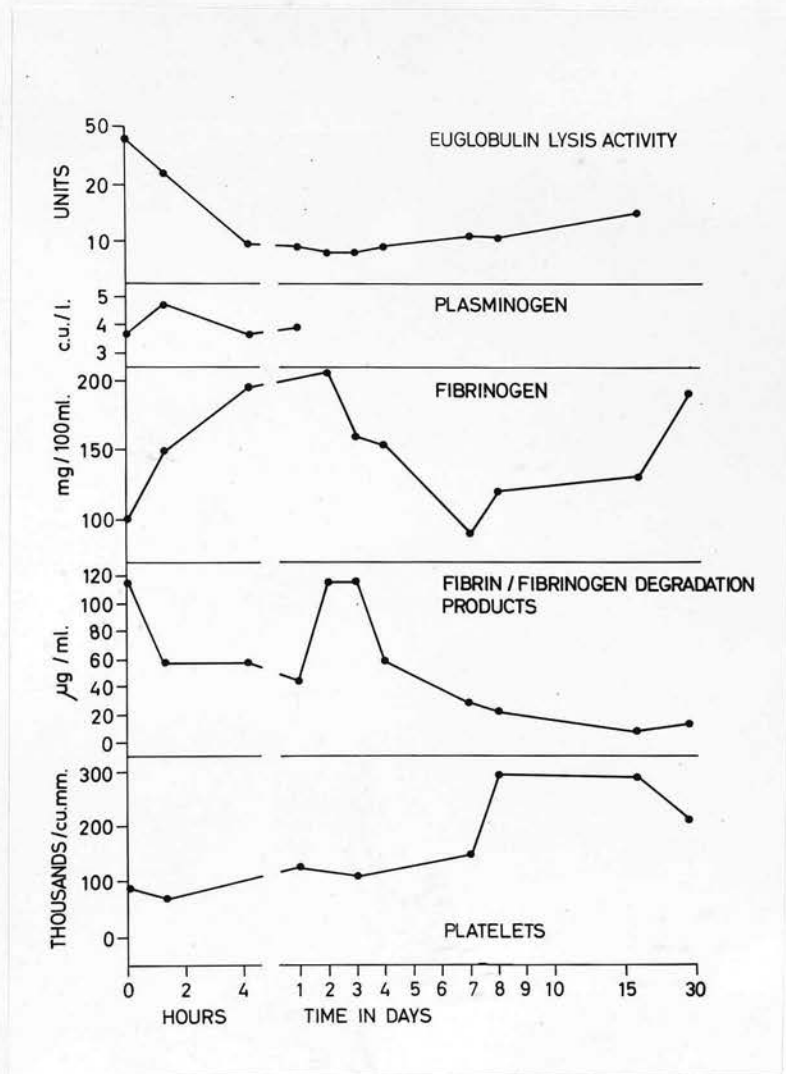


Fig. 5:27. Results of laboratory investigations on Mrs. M.S. (post partum haemorrhage).

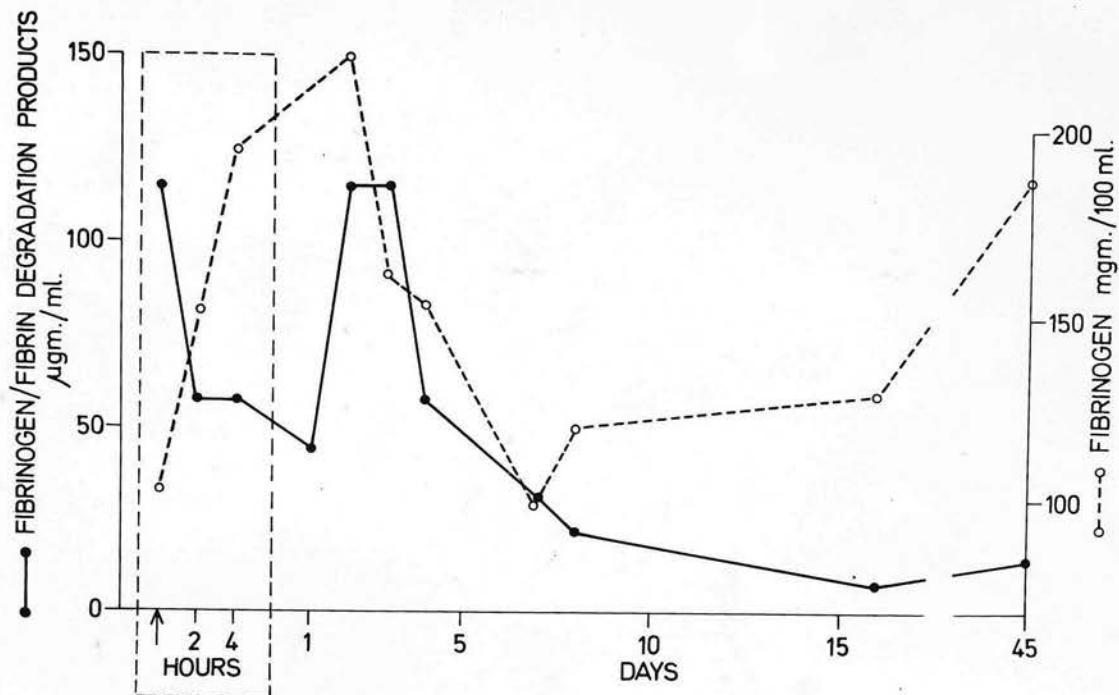


Fig. 5:28. Results of Fibrinogen/Fibrin Degradation Products and Fibrinogen Assays on Mrs. M.S. (post partum haemorrhage).

TABLE 5:29.

RESULTS OF LABORATORY INVESTIGATIONS ON MRS. M.S.

Day	Hours	Euglobulin Lysis Activity (Units)	Fibrin Plates (mm ²)		Plasminogen c.u./ml.	Fibrinogen (mgm/100 ml.)	F.D.Ps. μgm/ml.	Platelets per cmm.
			Euglobulin	Plasma				
1	0	44.80	841	333	3.74	103	115.0	86,000
-	2	26.50	-	225	4.70	151	57.6	70,000
-	4	-	-	-	3.70	195	57.6	-
2		8.55	778	361	3.90	-	43.2	125,000
3		8.02	613	255	-	210	115.0	-
4		8.22	638	138	-	162	115.0	110,000
5		8.97	-	-	-	153	57.6	-
7		10.80	587	187	-	100	30.0	145,000
8		10.40	695	170	-	121	21.6	290,000
16		14.77	489	-	-	129	7.2	285,000
30		-	-	-	-	187	14.4	210,000

treated with phenylbutazone.

The commonest causes of secondary post-partum haemorrhage are retention of a portion of the products of conception, or infection. In this patient there was very little tissue found on careful curettage of the uterus, although some enlargement of the uterus was noted. Infection was suspected although no definite evidence of this was available, i.e. the patient was afebrile, there had been no excessive lochial discharge, uterine tenderness was not marked, and a high vaginal swab was negative. A pregnancy test was also negative. A common side effect of phenylbutazone therapy is thrombocytopenia, but in this patient, although platelet levels were decreased, they were not reduced to a level at which bleeding usually occurs. An exact diagnosis thus remains elusive.

However, there is little doubt from the laboratory findings that this patient had good evidence of excessive fibrinolytic activity. Levels of F.D.P. were elevated and there was a marked decrease in the concentration of fibrinogen which is usually normally elevated in the post partum period (Rathoff et al., 1954; Shafer et al., 1968). There was also evidence of increased plasma proteolytic activity, either from increased levels of plasminogen activator or plasmin. The decrease of platelets is consistent with active intravascular coagulation although the reservation must be made that the drug therapy may also have affected this haemostatic parameter.

It is conceivable that this patient may have developed the rare syndrome described by Merskey et al. (1967) as idiopathic primary pathological fibrinolysis. These patients do not appear to have any specific provoking agent demonstrable for the activation of coagulation and exhibit hypo-

fibrinogenaemia coupled with marked evidence of clot or fibrin lysis in in vitro assays. There is usually a lesser disturbance of the coagulation system and the presentation of this patient could be consistent with this picture. However, separation of fibrinolysis into primary and secondary forms may not necessarily be valid and may only hide our ignorance of the basic etiology and mechanisms of coagulation occurring in this type of clinical situation.

The concentration of F.D.P. initially fell after the E.A.C.A./fibrinogen/blood therapy and by the following day levels were approaching the normal range. However, by the following day F.D.P. again increased to the pre-treatment level and remained high for the next three days. As these specimens were assayed in retrospect this was an unexpected finding, but repeat assays confirmed these changes. At the same time changes in fibrinogen levels occurred with a marked fall in concentration. This fall was only reversed when F.D.P. levels had fallen close to the normal range (Fig. 5:28). At about the time the fibrinogen was at its lowest level a small vaginal bleed occurred but this was not regarded as clinically important. It is thus probable that the provoking stimulus for the D.I.C. was of a continuous low grade nature, resulting in a slow incomplete defibrination of the patient, but not proceeding to a level which was clinically overt.

In retrospect, the use of heparin to decrease the rate of intravascular coagulation may have been a more reasonable treatment in this patient, particularly in view of the history of thrombophlebitis. Although the aetiological nature of the fibrinolytic stimulus remains obscure, this study

points to the importance of careful follow-up of patients who exhibit features of D.I.C., for not only may they occasionally develop thromboemboli phenomena in the convalescent phase but the fibrinolytic process may apparently continue in some patients in an undetected form.

DISCUSSION.

The recognition that hypofibrinogenaemia may be an important factor in haemorrhagic states associated with pregnancy has stimulated a great deal of fundamental research into clotting and lytic mechanisms. Although based largely upon animal experimental work the concept of disseminated intravascular coagulation being relevant in human disease has considerably extended both the laboratory and clinical approach to disorders of haemorrhage and thrombin, and now, perhaps for the first time, clinicians engaged in treating these problems are gaining an integrated picture of these two complex haemostatic systems.

It is apparent that disseminated intravascular coagulation, particularly in the obstetrical situation, is but an intermediary mechanism of the disease and we are still largely unaware of the basic processes whereby the coagulation system is initially stimulated. Treatment thus must be aimed at the management of laboratory-detected coagulation and fibrinolytic abnormalities in conjunction with conventional therapy directed towards correction of the underlying disease process. Treatment of individual patients must therefore be tailored to their particular needs and no standard therapy is as yet available. Indeed, it may be difficult to decide immediately on the most appropriate therapy and clinical experience

of comparable situations can be invaluable.

In the case reports presented in this chapter it can be seen that syndromes of D.I.C. can be identified specifically in a variety of obstetrical complications. It might be conjectured that the subjects who develop D.I.C. are included in the small group of individuals whose fibrinolytic response to stress is normally very low, and is decreased even further in pregnancy. There is no evidence to date to substantiate this latter theory but a follow-up and assessment of the fibrinolytic reactivity in patients who have exhibited the features of D.I.C. might be informative.

Previously, the substantiation of the diagnosis of D.I.C. has depended on comparatively non-specific coagulation tests, but using the recently developed rapid assay for F.D.Ps. it is now possible for the coagulation laboratory to state clearly if lysis of fibrin is excessive. Such information is clearly of the utmost value to the clinician and further close investigation of the use of this assay in obstetrical situations is indicated. A significant laboratory advance would be a simple and quick method for the identification of excessive thromboplastic-like activity in plasma, for this information might be indicative of a need to use anti-coagulant drugs rather than replacement of coagulation factors.

One of the many questions which remains to be answered is "Why do only a small proportion of 'at risk' subjects in pregnancy develop haemostatic problems?" The answer is clearly not a simple one. Hjort and Rapaport (1965) point out in their review on the Shwartzman reaction that normal animals can cope with extensive disseminated intravascular coagulation with few ill effects. However, alterations in reticulo-endothelial activity,

granulocytic activity, blood flow, immunity status and fibrinolysis, may modify the body's response to the stress with the appearance of Shwartzman-type reactions. In pregnancy fibrinolytic activity, as measured by plasminogen activity, is decreased, as is also the ability of some pregnant women to respond to a stress such as exercise. If superimposed on this, other factors such as those noted above and others as yet unknown are added, the combination of circumstances may be enough to allow the complications of disseminated intravascular coagulation to develop. This may only occur in a few patients, possibly explaining why only a small minority of subjects exhibiting features of D.I.C. develop severe haemorrhagic or thrombotic phenomena.

These reports are of particular interest as well documented cases of disseminated intravascular coagulation using a battery of assay systems are uncommon in the literature. The adequate laboratory investigations and satisfactory clinical outcome are a reflection of close liaison between clinical and laboratory staff.

SUMMARY.

The case histories of six patients, each exhibiting the features of D.I.C. and of different primary aetiologies, are presented.

The diagnosis and treatment of these patients are discussed in the light of presently available knowledge of the coagulation and fibrinolytic systems.

FINAL COMMENTS

Although in recent years the evolution of our knowledge of haemostatic mechanisms has been progressive, there still remain large gaps in our basic concepts of these systems. One important reason for this is the problem of methodology. In many instances the laboratory procedures developed for the measurement of parameters of coagulation and fibrinolysis have lacked specificity, accuracy or reproducibility, or have been designed solely for clinical recognition of major haemostatic pathology. The application of such methods to physiological studies has not been completely satisfactory, as parameter changes are often small and outside the sensitivity of the laboratory techniques. Coagulation assay techniques are a typical example of this problem as further standardisation of present methodology, relying as it does on the detection of fibrin end points, would appear unlikely to increase assay precision substantially.

Experience with conventional coagulation assay techniques reported in the present studies soon established that their application to physiological measurements of small, but perhaps important, homeostatic changes in coagulation was strictly limited. Numerous variables were inevitably present and despite careful standardisation, consistent and accurate reproducibility of results was not easy to maintain, this being particularly noticeable in measurements in the normal and supra-normal ranges of each clotting factor. However, it was possible to confirm some of the coagulation studies from other centres, thus implying that measurements were being made of similar biochemical entities. The changes occurring in Factor VIII for instance, both with exercise and

adrenalin, were clear cut, but on the other hand the results of the Factor XII assays were dissimilar to one other group of workers. With new more specific assay techniques it might be possible to resolve such differences and measure accurately small differences in concentration.

Because of the need to increase our understanding of the various inter-actions of these important enzyme systems coagulation and fibrinolysis are now being investigated by techniques previously allied to other disciplines. Sensitive biochemical and immunological methods have, for instance, contributed considerably to our insight of sequential molecular events occurring in the proteolysis of fibrinogen by thrombin and plasmin. With the development of immunological techniques for the identification and quantitative assay of fibrin/fibrinogen degradation products, a new approach is now possible to certain aspects of the haemostatic mechanism.

It would appear that the use of immunological assays in coagulation and fibrinolytic studies is probably only commencing. As specific components of the haemostatic system become biochemically purified and concentrated, it will become increasingly possible to produce monospecific antisera and, coupled with haemagglutination, radio-immunoassay and other techniques, further elucidation will be possible in both physiological and pathological studies. Measurement of coagulation Factor XII has already been adapted to a haemagglutination assay (Smink et al., 1967) and Factor II can be assayed by an immunochemical method (Nilehn and Ganrot, 1968). With development of the serological work reported by Denson (1967) basically similar measurements of other clotting factors may also be feasible. These techniques are also applicable to fibrinolysis, as demonstrated by the studies of Kucinski et al.

(1968) who have developed an antisera to purified urokinase. Future investigatory procedures thus appear most promising and major advances in our fundamental knowledge can reasonably be expected.

Partly because of the limitations of techniques it was not possible to extend appreciably the concept of a dynamic balance existing between fibrin formation and lysis. Alterations in absolute levels of coagulation factors could not be related to changes in fibrinolytic parameters, and although the measurement of cryofibrinogen was of interest, the specificity of changes in its concentration is still not clear. The demonstration of F.D.P. in nearly all sera examined might be regarded as definitive proof of a continuous intravascular balance but, as we have indicated earlier, these polypeptides may be derived from extravascular sources (Das et al., 1968) and much more information is required before such a concept could be accepted. Perhaps a dynamic equilibrium could be assessed more adequately if measurements of either the in vivo changes of fibrin monomer or the peptides released on thrombin proteolysis of fibrinogen and fibrin could be made. In this thesis, various changes in cryofibrinogen in the form of heparin precipitable protein have been demonstrated but the detection of cryoprofibrin (Shainoff, 1962) in normal plasma might be a more relevant measure of fibrin monomer. The development of a reliable immunological measurement of fibrinopeptides A and B might also be an advance in our interpretation of the theory and some work is already in progress (Berglund and Blombäck, 1965). Nevertheless, the demonstration of these products in normal plasma would still not be final proof of such an equilibrium as there would be a need to establish the contribution made by extravascular sources to the intravascular pool. It

might, however, be theoretically possible to equate fibrin formation and lysis by measuring simultaneously the response of the coagulation and fibrinolytic peptides to stimuli regarded as activating both these systems. Sensitive methods for measuring fibrinogen turnover could also reveal much relevant to physiological haemostasis. Thus much depends on improved methodology, and research in the next few years should uncover much of value.

A concept frequently noted in this thesis is that of the "fibrinolytic reactivity" of the plasminogen activator system. The relevance of this concept to pathophysiological events is not yet known, and will probably not be, until more is known of the exact in vivo role of plasminogen activator coupled with an understanding of its origin, metabolism, inhibition and excretion. The measurements of this labile enzyme depends upon in vitro systems that greatly magnify its lytic potential, after prior removal or diminution of naturally occurring inhibitor activity. Although circulating plasminogen activator may be the component responsible for routine fibrin lysis, a real need will be fulfilled when a rapid overall assessment of blood fibrinolytic activity is available and it might then be possible to assess more completely the physiopathological importance of changes in the components of this enzyme system. We do not understand, for instance, the significance of prolonged euglobulin lysis times occurring in apparently healthy subjects, or the relevance of the varying levels of plasminogen activator between individuals, not only within and between days but also in response to diverse stimuli. It is probable that these questions will remain unanswered until further epidemiological, physiological and pathological studies are carried out using improved methodology. A strong possibility exists that measurement

of activator levels represents only one factor in continually and dynamically changing mechanism, and that a realistic picture can be obtained only when other parameters, such as the various fibrinolytic inhibitors, are simultaneously measured.

Pathological fibrinolytic activity in pregnancy is a not uncommon clinical entity and as its effects on maternal and foetal mortality are marked, investigations of its pathophysiological variations are of some importance. However, although it is now possible to measure the quantitative changes in some coagulation and fibrinolytic parameters, such information raises only fresh problems of interpretation. Insight into the significance of these changes and particularly their inter-action, control, and metabolism still completely evade us. Are these alterations of teleological significance in haemostasis or are they representative of non-specific responses to stress? To what extent do these systems integrate to maintain vessel patency in the vascular bed of the placenta and do placental metabolic products modify this process? The many questions which arise serve only to emphasize our incomplete concepts of these systems and provide considerable room for further research. Nevertheless, further investigation in pregnancy of the concept of fibrinolytic reactivity might provide an alternative approach to the study of clinically evident Schwartzman-type reactions and their associated haemostatic changes. There is a need to establish if this phenomena plays a major or minor part in the bleeding or clotting problems of pregnancy and to develop simpler methods of assessing fibrinolytic reactivity.

Fibrinolysis cannot simply be equated with changes in plasminogen activator levels since lysis of fibrin, as measured by the F.D.P. assay, may

be quite unaltered. The study of F.D.P. in pregnancy perhaps emphasizes this point. Low plasminogen activator levels cannot simply be linked with low fibrinolytic activity and theories based on the sole measurement of this parameter in vascular disease will require to be reviewed. Doubts as to the value of the observation of the speed of lysis of euglobulin clots has been expressed by Jacobsen (1968) who, in a study of the proteolytic capacity of plasma within family groups, was not able to show that greatly decreased fibrinolysis predisposed towards thrombosis.

Measurement of the coagulation and fibrinolytic changes occurring in the operative and post operative phases will gradually reveal more of the mechanisms of bleeding and thrombotic phenomena. Certain lines of investigation have already been suggested in this thesis but such studies will be of less value unless other relevant parameters, such as fibrinogen turnover and platelet function studies, are assessed simultaneously. It is almost certain that the background to many haemostatic emergencies is multifactorial in origin and adequate investigation will demand integration of specialist investigations, in combined, projects, to achieve the maximum advance in knowledge. Perhaps the paramount problem is the early diagnosis of pre-thrombotic states. Conventional coagulation and fibrinolytic techniques have not contributed greatly to the understanding of this phenomena and there is a need to orientate new assay techniques towards the investigation of thromboembolism. Confirmation and extension of the post operation findings reported in this thesis might have a direct effect on the management of selected patients in the post operative phase, although much more exacting study is required.

Specific identification of many abnormal states of fibrin lysis can now be made using the F.D.P. assay. It can be shown that excessive lytic activity is possible in the absence of significant haemorrhage and the development of rapid F.D.P. assays now allows early confirmation of suspected fibrinolytic problems. A rapid method for the identification of the earlier products of fibrinogen proteolysis by plasmin would be a useful advance, as would be a more specific method for measuring the varying sized degradation products. However, the F.D.P. assay, especially in obstetrical pathology, should, even at the present time, prove a valuable ancillary routine diagnostic tool in the coagulation laboratory. It will now be possible to define more clearly the significance and frequency of syndromes of disseminated intravascular coagulation.

The challenges still existing in the biochemical unravelling of these complex and integrated systems and the elucidation of their relationships to haemorrhage and thrombosis still remain, but it can confidently be expected that the new and developing laboratory techniques will answer many of the fundamental problems outlined in these studies. Such elucidation might be of considerable significance in many distinct areas of clinical medicine.

APPENDIX

A. Collection and Storage of Blood Specimens.

Blood specimens were normally collected from an antecubital vein by careful venepuncture using the minimum of venous occlusion of the arm. All venepunctures were performed by experienced medical staff and where there was difficulty entering a vein the specimen was discarded. Plastic syringes and disposable needles (gauge size 20 or 21) were used throughout the studies. After collection the blood was carefully mixed, without frothing, with the anticoagulant or protease inhibitor. For some tests, e.g. the euglobulin lysis time assay and some clotting tests, the blood was stored for short periods on melting ice.

After centrifugation of the blood samples, plasma was separated using pasteur pipettes and either assayed immediately or stored in small aliquots at -40°C .

B. Statistics.

Routine statistical computations were made on the Olivetti 100 Desk Computer with programmes which included the following mathematical formulae.

(a) Standard Deviation

$$S.D. = \sqrt{\frac{N \sum x^2 - (\sum x)^2}{N(N-1)}}$$

Where x = variate value

N = number of values.

(b) Coefficient of Linear Correlation and Rate of Regression.

The calculation was based on the Bravais-Pearson coefficient of linear correlation.

$$r = \frac{N \sum xy - (\sum x)(\sum y)}{\sqrt{[N \sum x^2 - (\sum x)^2][N \sum y^2 - (\sum y)^2]}}$$

Where x and y = variate values

N = number of observations.

The probability (p) of x and y being correlated was obtained from correlation coefficient tables where the degrees of freedom were equal to $N - 1$. The programme also computed the parameters a and b of the regression lines between the two variables x and y .

$$y = a + bx$$

$$b = \frac{N \sum xy - (\sum x)(\sum y)}{N \sum x^2 - (\sum x)^2}$$

$$a = \frac{\sum y - b \sum x}{N}$$

Where x and y = variate values

N = number of observations.

The results were plotted by inserting the values for $a + b$ into

for formula $y = a + bx$, and drawing the regression line for arbitrary values of x .

(c) Students t test for significance.

(i) The value of t was computed to ascertain whether the mean of two samples, taken from the same populations, differ significantly.

$$M_1 = \frac{\sum x}{N_1}$$

$$M_2 = \frac{\sum x}{N_2}$$

$$S_1^2 = \frac{N_1 \sum x^2 - (\sum x)^2}{N_1(N_1 - 1)}$$

$$S_2^2 = \frac{N_2 \sum y^2 - (\sum y)^2}{N_2(N_2 - 1)}$$

$$\text{Standard Deviation} = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}$$

$$t = \frac{M_1 - M_2}{\text{S.D.} \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Where M_1 = arithmetic mean of first sample

M_2 = arithmetic mean of second sample

S_1^2 = variance of first sample

S_2^2 = variance of second sample

N_1 = number of values in first sample

N_2 = number of values in second sample

x and y = variate values.

The p (probability) was obtained from a Students t - distribution table, with the degrees of freedom being equivalent to $N_1 + N_2 - 2$. Values of p equal to or less than 0.05 were regarded as being significant.

(ii) This additional t test was used for testing for significance between paired samples from the same subject. One mean calculated from the sum of the pair differences was tested.

$$t = \frac{\bar{x}}{\sqrt{\frac{SD^2}{N}}}$$

$$SD^2 = \sqrt{\frac{\sum d^2 - \frac{1}{N} (\sum d)^2}{N(N-1)}}$$

Where N = number of samples

d = difference between pairs

\bar{x} = mean of sample of measurements x.

The significance of the value t was obtained from t test tables, where the degrees of freedom was equal to the number (N) of paired samples. Values equal or less than 0.05 were regarded as significant.

Statistical tables used were obtained from either Documenta Geigy, "Scientific Tables", 6th Edition 1962, or from "Statistical Methods in Biology" by N.T.J. Bailey (1959).

(d) Mathematical formulae for computer programme

These mathematics were used for calculation of potency ratio and confidence limits between test and control plasma clotting times and are

as prepared for the K.D.F. - 9 Computer*. These mathematical procedures can be equated with the text of Section 3:5, "Coagulation and the Computer".

(i) Calculation of regression equations for both test and control plasma clotting times.

$$b_1 = \frac{\sum (x_1 - \bar{x}_1) (y_1 - \bar{y}_1)}{(x_1 - \bar{x}_1)^2}$$

$$b_2 = \frac{\sum (x_2 - \bar{x}_2) (y_2 - \bar{y}_2)}{(x_2 - \bar{x}_2)^2}$$

Where b_1 = slope of control plasma

b_2 = slope of test plasma

$x_1 + x_2$ = log dilutions at which plasmas tested

$y_1 + y_2$ = clotting times patient's plasma

$\bar{y}_1 + \bar{y}_2$ = mean clotting times control plasma

\sum = summation over all readings.

Each regression is then tested for linearity. Only dilutions (x) with 2 or more clotting times (y) readings can be used in the computation of k (the number of dilution readings) must be not less than 3. A standard F test (analysis of variants) is used.

$$F = \frac{S_2^2}{S_1^2}$$

$$S_2^2 = \frac{\sum_{i=1}^k m_i (y - Y_i)^2}{k - 2}$$

$$S_1^2 = \frac{\sum_{i=1}^k S Y_i}{N - k}$$

Where k = number of x readings

m = number of y readings

Y_i is calculated from regression equation

N = number of values included in computation.

(ii) Test for homogeneity of variance.

(a) The variances about the regression lines having been computed the F test is again applied as an indication of any differences between variances of the test and control plasmas.

$$\text{Variance} = \frac{SY^2 - bSXY}{n - 2}$$

$$\text{Where } SXY = \frac{\sum xy - \sum x. \sum y}{n}$$

$$XY^2 = \frac{\sum y^2 - (\sum y)^2}{n}$$

$$SX^2 = \frac{\sum x^2 - (\sum x)^2}{n}$$

$$b = \frac{SXY}{SX^2}$$

n = number of readings in experiment.

The pooled residual variance (V_r) is then calculated.

$$V_r = \frac{1}{n_1 + n_2 - 4} \left\{ \left[\sum (y_1 - \bar{y}_1)^2 - \frac{\{ (\sum x_1 - \bar{x}_1)(y - \bar{y}_1) \}}{(x_1 - \bar{x}_1)^2} \right] + \right. \\ \left. \sum \left[\sum (y_2 - \bar{y}_2)^2 - \frac{\{ \sum (x_2 - \bar{x}_2)(y_2 - \bar{y}_2) \}}{(x_2 - \bar{x}_2)^2} \right] \right\}$$

The standard error of the difference between the two slopes is computed from the formula :-

$$S.E.(b_1 - b_2) = \sqrt{Vr \left(\frac{1}{\sum (x_1 - \bar{x}_1)^2} + \frac{1}{\sum (x_2 - \bar{x}_2)^2} \right)}$$

(b) Application of t test.

$$t = \frac{b_1 - b_2}{S.E.(b_1 - b_2)}$$

$$\text{Degrees of freedom (df)} = n_1 + n_2 - 4$$

The probability of obtaining by chance as great or greater a ratio is obtained from a t test table.

(iii) If the dose response curves are not significantly non-parallel the log potency ratio (M) is obtained :-

$$M = \bar{X}_2 - \bar{X}_1 - \left(\frac{\bar{Y}_2 - \bar{Y}_1}{B} \right)$$

$\bar{X}_1 + \bar{X}_2$ = mean tested concentrations of the two plasmas in real log. units.

$\bar{Y}_1 + \bar{Y}_2$ = mean responses for the two plasmas in real (or transformed units) of response.

B = common slope obtained from the formula :-

$$B = \frac{\sum (x_1 - \bar{x}_1)(y_1 - \bar{y}_1) + \sum (x_2 - \bar{x}_2)(y_2 - \bar{y}_2)}{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}$$

The potency of the patient's plasma in terms of the control plasma is given by antilog M.

(iv) The Standard error of the log potency ratio is calculated :-

$$S.E.(m) = \sqrt{\frac{\frac{Vr}{B_2} \frac{1}{n_1} + \frac{1}{n_2} + \left(\frac{\bar{Y}_2 - \bar{Y}_1}{B}\right)^2}{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}}$$

(Pooled residual variance (vr) is calculated from $n_1 + n_2 - 3$ degrees of freedom at this stage.)

The 95% confidence limits of the log potency ratio

$$= M \pm SE(m) \times t_{0.05} (v = n_1 + n_2 - 3)$$

By taking antilogs., the limits are then obtained as percentages of the antilog. of M.

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C. Clinical Details of Surgical Patients.

(a) Caesarian Section Group.

C.S. 1. Mrs. M.K.; age 20; Para. 1.

History of an unstable lie at 39 weeks and contracted pelvis.

An abnormal glucose tolerance test curve was noted.

Lower Segment Caesarian Section (L.S.C.S.) was performed with delivery of a live babe (wt. 10 lbs.). No post operative complications.

C.S. 2. Mrs. E.O.; age 36; Para. 2 + 1.

History of small antepartum haemorrhage at 38 weeks. Two previous pregnancies had been uneventful; medical history normal.

L.S.C.S. for Type 1 placenta praevia at 38 weeks with delivery of live babe. At operation there was numerous adhesions involving uterine appendages and bowel. One part of blood was transfused during the operation. Given two bottles of packed cells post operatively for anaemia and Cycloserine for a urinary tract infection. No other complications.

C.S. 3. Mrs. M.F.; age 25; Para. 2 + 0.

Admitted because of Rhesus immunisation. Intra-uterine transfusions had been performed at 24, 28 and 31 weeks. Medical history otherwise normal.

L.S.C.S. performed at 34 weeks. Live but severely affected male babe delivered. Post operative course was uneventful.

C.S. 4. Mrs. G.McM.; age 22; Para. 3 + 0.

History of three previous Caesarian Sections for contracted pelvis. Medical history otherwise uneventful.

L.S.C.S. at 38 weeks with delivery of a live babe. Post operative course was normal.

C.S. 5. Mrs. I.R.; age 42; Para. 5 + 1.

Admitted to hospital at 36 weeks because of unstable lie. No significant medical or obstetrical history.

L.S.C.S. at 38 weeks with delivery of a live babe. Bilateral tubal ligation was also performed. At operation the placenta was inadvertently incised as uterus was being opened. Operation otherwise uneventful. Post operative period uncomplicated.

C.S. 6. Mrs. R.P.; age 24; Para. 3 + 0.

Admitted for suspected Rh disease, with high Anti (C+D) titres. Previous pregnancies had been uneventful and there was no other significant medical history.

L.S.C.S. was performed at 34 weeks with delivery of a live babe severely affected with Rhesus immunisation. Post operative course uneventful.

C.S. 7. Mrs. A.L.; age 26; Para. 1 + 2.

Previous history of Caesarian Section for contracted pelvis. Two previous miscarriages. Medical history uneventful.

L.S.C.S. performed at 39 weeks, with bilateral tubal ligation. A live babe was delivered. The placenta was removed manually. Post operative course uneventful.

C.S. 8. Mrs. E.A.; age 34; Para. 0.

Previous history of infertility for 6 years. Admitted for hypertension occurring near term, associated with a slight antepartum haemorrhage which settled on conservative treatment.

L.S.C.S. was performed at 42 weeks as an emergency, for foetal

distress, with delivery of a dead babe (cord round neck). Slight pyrexia post operatively, attributed to chest infection, otherwise no other complications.

C.S. 9. Mrs. E.M.; age 36; Para. 3 + 0

Admitted for elective Caesarian Section at 39 weeks after previous pelvic floor repair. Medical history satisfactory.

L.S.C.S. and tubal ligation was performed. A live babe was delivered. Post operative course uneventful.

C.S. 10. Mrs. C.C.; age 34; Para. 2 + 1.

History of an unstable lie at 39 weeks. Also had had a previous Caesarian Section.

L.S.C.S. performed with delivery of a live babe. Uneventful post operative course.

C.S. 11. Mrs. J.S.; age 39; Para. 0 + 1.

Previous history of infertility and a recent miscarriage. Admitted for ? post maturity, excessive weight gain and maternal age.

Elective L.S.C.S. performed at 38 weeks by dates, with delivery of live babe. Post operative course uneventful.

C.S. 12. Mrs. M.S.; age 28; Para. 2.

This patient had had two previous Caesarian Sections for a contracted pelvis.

Elected L.S.C.S. performed with tubal ligation, and delivery of a live babe. Post operative course was complicated by a chest infection and a urinary tract infection. Both responded to Ampicillin.

C.S. 13. Mrs. R.M.; age 24; Para. 4 + 3.

Admitted at 39 weeks gestation for elective Caesarian Section following previous section.

L.S.C.S. performed with tubal ligation. Live babe born. Post operative course complicated by a mild chest infection responding to Ampicillin.

C.S. 14. Mrs. A.C.; age 40; Para. 0.

History of failed external cephalic version of breech position at 33 weeks. Admitted for elective Caesarian Section in view of age and breech presentation.

L.S.C.S. performed with delivery of live babe. No complications. Post operative course uneventful.

(b) Intra-abdominal Gynaecological Operation Group.

H. 1. Mrs. M.A.; age 42.

Admitted with a history of a mass in the lower abdomen for six months associated with heavy periods.

A total hysterectomy, but leaving one ovary, was performed. A large uterine fibroid was found. Post operative course uneventful.

H. 2. Mrs. H.McL.; age 39.

Admitted following laboratory report of a suspicious cervical smear.

At operation a partial hysterectomy was performed. Pathology of the cervix showed a small carcinoma - in situ. Post operative course uneventful.

H. 3. Mrs. B.S.; age 34.

Admitted after a history of six months menorrhagia.

At operation a total hysterectomy was performed. Pathology of the uterus was normal. Post operatively she developed a pyrexia and a vaginal vault haematoma. Treated with Ampicillin and made a slow recovery.

H. 4. Mrs. E.E.; age 28.

Admitted for a ventro suspension operation for retroversion of the uterus for dyspareunia. This was performed along with salpingectomy and appendicectomy.

Post operatively a chest infection developed which settled with Penicillin. Two weeks after the operation she was again admitted with a chest infection which settled without further treatment.

H. 5. Miss A.B.; age 35.

Admitted for investigation of irregular period and dysmenorrhoea. No other medical history, apart from congenital eye and skeletal defects since birth.

A total hysterectomy was performed. Nil abnormal found. Post operative course satisfactory.

H. 6. Mrs. D.S.; age 32.

Admitted for investigation of menorrhagia. Nil abnormal found clinically.

At operation a partial hysterectomy was performed. The uterus was bulky but otherwise normal. Post operative course uneventful.

H. 7. Mrs. J.B.; age 30.

History of two previous Caesarian Sections for contracted pelvis; also recent development of a depressive illness. Recommended for sterilization by G.P. No other significant medical history.

Bilateral salpingectomy was performed. Post operative course was uneventful.

H. 8. Mrs. L.W.; age 43.

History of lower abdominal mass for six months.

At operation large fibroids were found. A total hysterectomy was performed. Developed an infected haematoma post operatively which settled without specific treatment.

H. 9. Mrs. A.C.; age 41.

Admitted for investigation after suspicious cervical smear laboratory report.

Total hysterectomy and left salpingoophorectomy performed. No evidence of carcinoma on histological examination of the cervix. Post operative course was satisfactory.

H. 10. Mrs. J.M.; age 33.

History of a deep vein thrombosis five years previously. Since then had had left leg pain associated with periods. More recently developed menorrhagia.

At operation a subtotal hysterectomy was performed. Three days post operatively she developed left leg pain and tenderness. Treated as a deep venous thrombosis with heparin and anticoagulants and these were continued for four weeks. For a urinary infection she was given Ampicillin. Also sustained a vaginal haematoma with consequent anaemia. Made a slow recovery and discharged from hospital on anticoagulants.

H. 11. Miss E.T.; age 21.

History of a lower abdominal mass for six months with polymenorrhoea.

No other medical history of note.

At operation a large cystic left ovary found. Right salpingoophorectomy performed. Post operative course satisfactory.

D. General Reagents.

1. Buffers.

(a) Tris buffer pH 7.8.

73 gm. of tris hydroxymethyl aminomethane (Koch Light Laboratories) was dissolved in 2 litres of distilled water and the pH adjusted to 7.8 with N Hydrochloric acid. Solution then made up to 4 litres with distilled water and the pH checked.

(b) Veronal acetate buffer pH 7.4.

9.714 gm. of sodium acetate trihydrate and 14.714 gm. of sodium diethylbarbiturate were dissolved in 500 ml. of distilled water. This stock solution was stored at 4°C.

A working solution was made by fixing 25 ml. of stock veronal acetate buffer and 25 ml. of 0.1 N HCl and the volume made up to 500 ml. with 0.9% saline. The pH was adjusted to 7.4 with either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. The solution was stored at 4°C.

(c) Glyoxaline (Imidazole) buffer (Mertz and Owen, 1940).

680 mg. of glyoxaline (Koch Light Laboratories) was dissolved in 50 ml. of distilled water. The buffer at pH 7.3 was prepared by mixing 2.5 parts of this base with 1.86 parts of 0.1 N hydrochloric acid, and 5.64 parts of distilled water. To each 100 ml. of buffer was added 0.585 gm. of sodium chloride. The buffer was stored at 4°C.

(d) 0.05 ml. Tris buffer pH 7.2.

24.30 gm. of tris buffer (Koch Light Laboratories) was dissolved in 1 litre of distilled water. From this stock solution 250 ml. was added to 42 ml. of N hydrochloric acid and the volume made up to 940 ml. with distilled water. The pH was adjusted to 7.4 at room temperature (20°C).

(e) 0.1 M Barbitone-saline buffer pH 7.22.

5.71 gm. of sodium barbitone and 2.93 gm. of sodium chloride were added to 960 ml. of distilled water and the pH adjusted to 7.22. The volume was then made up to 1 litre with distilled water.

(f) 0.1 M Phosphate buffer pH 7.6.

13.6 gm. of potassium dihydrogen phosphate was dissolved in 1 litre of distilled water. 14.2 gm. of disodium hydrogen phosphate was similarly made up to 1 litre with distilled water. 870 ml. of the former and 130 ml. of the latter was then mixed and the pH checked.

2. Other Reagents.

(a) Sodium hydroxide 0.5 N.

10 gm. of sodium hydroxide was dissolved in 500 ml. of distilled water.

(b) Sodium hydroxide 10%.

50 gm. of sodium hydroxide was dissolved in 500 ml. of distilled water.

(c) 5% Trichloroacetic Acid.

25 gm. of trichloroacetic acid were dissolved in 500 ml. of distilled water.

(d) Tannic Acid.

A stock solution is prepared by adding 100 mgm. of tannic acid to 10 ml. of distilled water.

(e) Acetic acid for Euglobulin Lysis Time Estimations.

5 ml. of glacial acetic acid was added to 500 ml. of distilled water. 19.5 ml. of this 1% solution of acetic acid was added to 2 litres of distilled water for use in the E.L.T. estimation.

(f) 3% Formal Saline.

30 ml. of Formaldehyde solutions (Evans) was added to 1 litre of 0.9% saline and the pH adjusted to 7.25 with N. NaOH.

(g) Calcium Chloride Solutions.

0.05 M and 0.025 M calcium chloride solutions were made from a stock solution of M Calcium chloride (British Drug Houses Ltd.)

(h) Solution of Epsilon Amino Caproic Acid (E.A.C.A.) for Fibrinogen Assay.

710 mgm. of E.A.C.A. was dissolved in 1 litre of distilled water.

(i) Dye for Fibrin Plates.

0.5 gm. of Bromothymol Blue powder was added to a mixture of 250 ml. of Ethyl alcohol and 250 ml. of 0.5 N sodium hydroxide.

E. Cleaning and Siliconization of Glassware.

1. Cleaning of Glassware.

(i) Test tubes and other glassware.

(a) Glassware immersed for at least 24 hours in strong

detergent solution of Pyroneg (Diversey U.K.)

- (b) Boiled in a solution of strong pyroneg for 30 minutes.
- (c) Washed thoroughly to remove all traces of detergent.
- (d) Immersed in chromic acid for a minimum of 24 hours.
- (e) Rinsed free from chromic acid in running water.
- (f) Washed with a minimum of three changes of hot water and three changes of cold water.
- (g) Final wash for three changes of distilled water.
- (h) Dried in hot air oven overnight.

(ii) Pipettes

After use, soaked for at least 24 hours in a dilute solution of Pyroneg; then rinsed for 2 hours with running hot water in an automatic pipette washer, followed by 2 hours using cold water. The final three washes were with distilled water. Pipettes were dried in a hot air oven overnight.

2. Siliconization of Glassware.

In initial experiments, glassware was siliconized by immersion in a 3% solution of I.C.I. M550 silicone in trichlorethylene for 2 hours; then dried in a hot air oven, rinsed at least six times in distilled water and re-dried overnight in a similar manner.

In later experiments all glassware was siliconised with "Siliclad" (Clay Adams, N.Y.) in a 1% solution. After siliconization, glassware was similarly washed and dried in a hot air oven.

F. MIDDLE-AGED VOLUNTEERS - ELECTROCARDIOGRAPH REPORTS

	1966	1967
M1	Normal	Normal
M2	"	"
M3*	"	Post exercise depression of ST segment indicative of ischaemia.
M4	"	Normal
M5	"	"
M6	"	"
M7	"	"
M8	"	"
M9	"	"
M10*	"	"
M11	"	"
M12	"	"
M13	"	"
M14	Right Bundle Branch Block Normal Otherwise.	Right Bundle, Branch Block. Normal Otherwise. No Change.
M15	Normal	Normal
F1	"	"
F2	"	Post exercise ischaemia changes present
F3	"	Normal

* Fibrinolytic poor responders.

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